# LABORATORY TECHNIQUE

FOR THE

# STUDY OF MALARIA


By

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Foreword by

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*WITH 25 ILLUSTRATIONS*




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## FOREWORD

The treatment of neurosyphilis by malariatherapy introduced in Austria by Wagner Jauregg in 1917 was first used in Great Britain in 1922

In the following year on the advice of Lt. Col S P James Adviser of Tropical Diseases to the Ministry of Health it was decided to provide facilities for the routine treatment of neuro syphilitic patients by malariatherapy throughout the United Kingdom From 1923 to 1925 batches of mosquitoes caught as adults from buildings in the low lying areas of Kent and Essex were fed on patients suffering from induced malaria in various hospitals mostly in the London area The mosquitoes were then taken to the Ministry of Health in Whitehall individuals from each batch being dissected from time to time until sporozoites were found in the salivary glands Requests from hospitals were then met by taking infected mosquitoes to them and allowing them to feed on the patient requiring malariatherapy

With the increasing demand for infective material this make shift arrangement proved unsatisfactory and it was then decided to establish a special malaria laboratory in the grounds of a mental hospital The Manor Hospital at Epsom which had been used for the treatment of tropical diseases during World War I was first considered as a location but since this was now occupied exclusively by mental defectives another hospital Horton situated in the same area was eventually selected A small villa in its grounds containing 12 beds was taken over as a treatment centre and two rooms in it were fitted up for laboratory work Mr P G Shute who had been James s assistant since 1918 and had previously worked in the laboratory of the Manor Hospital under James and Ronald Ross has been in charge of the Horton Laboratory since its inception

For the last 21 years he has been assisted by Miss M Maryon who has been co author with him of a number of scientific papers

In order to fulfil the functions of the laboratory it has been necessary not only to infect mosquitoes but to keep them alive for several weeks beyond the time when the salivary glands

become infected so that they may be transported to hospitals in any part of the country and used to *infect patients requiring* malariatherapy It has also been necessary to breed and maintain large numbers of mosquitoes in the insectaria so that they may be available at short notice when patients undergoing malariatherapy are found to have gametocytes in their blood

The practice of malariatherapy has rendered it possible to study in detail the natural course of primary attacks of malaria in non immune subjects and of relapses and a wealth of clinical material has become available for research in chemotherapy parasitology pathology and immunology Advances have also been *made in the study of mosquito ecology and of malaria* carrying properties of different species of mosquitoes from many parts of the world

These activities have involved the use of a large number of techniques both for the establishment and maintenance of laboratory colonies of mosquitoes and for the study of the malaria parasite in all its phases in the mosquito and the human host In some cases new techniques have been evolved while in others some modification of an existing technique has led to improve ment in the results obtained

*It is the purpose of this book to describe the techniques which* have been adopted for routine use in this laboratory The authors do not claim that all these are original but merely that they or the modifications introduced by them have proved useful in their hands They are aware that other methods are used by other workers which may be of equal value or even superior to those here described If they have not been mentioned it is either because the authors have not tried them or having tried them have not found them entirely satisfactory

I am confident that the techniques described in this book *representing as they do the results of 35 years* of experience of laboratory and field work will be of great practical value to students of malariology and to all those engaged in the study of insect borne diseases

GORDON COVELL

# PRFFACE

Tins book has been written as the result of numerous requests from workers in various parts of the world many of whom have been given facilities for study at various periods since our laboratory was established 33 years ago

During this period more than 13 000 patients have been infected with one or other of the four species of human Plasmodia involving the use of some 100 000 mosquitoes most of which were bred in the laboratory The monkey Plasmodium *P knowlesi* was also used to infect a limited number of patients during the period 1934–35

Pertinent references are cited at the end of each section and the titles of a number of other papers published from this laboratory which contain details of special techniques are listed at the end of the book

P G S
M M

## *ACKNOWLEDGEMENTS*

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## SECTION 1

### PREPARATION AND STAINING OF BLOOD FILMS

**Glass slides used for making blood films**

Slides known to the trade as half white are satisfactory 76 mm × 25 mm (3 in × 1 in) × 0·8–1 mm thick with ground smooth edges It is essential that the slides used for both thin and thick films should be grease free scratch free clean and dry If a slide is greasy a thin film will not spread evenly and a dried blob of blood will peel off a thick film during the process of staining We find that with new slides all that is required is to wipe them with a clean cloth and pass them through a mixture (equal parts) of ether and methyl alcohol For used slides we find that soaking them in 25% lysol for a few days removes the old film They are then thoroughly rinsed in hot water to remove all traces of lysol then soaked for a day or two in 1% teepol again thoroughly rinsed in hot water and dried with a clean grease free cloth Some slides retain their transparency for a long time but some become foggy and mottled with brownish patches which cannot be removed because the discoloration is in the glass and not on the surface these should be discarded

#### THIN BLOOD FILMS

**Technique of making thin films**

After cleaning *the pad of the finger or lobe of the ear the skin* should be wiped dry Puncture the skin with a straight triangular surgical needle and wipe off the first drop of blood A bead of blood for a thin film should be no larger than the head of a pin If a slide is used for spreading the film see that it is a thin one and has a smooth edge Contact of the slide with the bead of blood should be at an angle of 45 degrees and the film should be made by pushing the spreader away from the blood The aim in making a thin film should be to have the corpuscles only one deep the cells should almost touch one another but not overlap Good thin films cannot be made if a thick slide is used for spreading the film if the drop of blood is too large or if the blood has been

spread too rapidly The slide used as a spreader should have a smooth edge otherwise gaps will occur in the film

**Leishman stain**

METHODS OF PREPARATION

There are three methods of preparing the stain—

(1) 0 15 gm of dry crystals are placed in a mortar small quantities of methyl alcohol are added and pounded with the pestle and the supernatant fluid is poured into a clean dry bottle of hard glass with a *ground glass stopper Then more alcohol is added by* degrees until all the crystals are dissolved and finally made up to 100 ml Stain prepared in this way is ready for use within 24 hours Leishman stain should never be filtered

(2) *100 ml of methyl* alcohol is poured into the bottle and 0·15 gm of crystals added Several times throughout the day the bottle containing the stain is rocked to prevent the dye settling and sticking to the bottom of the container The stain is ready for use within 3 or 4 days

(3) 0 15 gm of crystals are *reduced to powder* with pestle and mortar Small quantities of methyl alcohol are added and the mixture is stirred with the pestle further methyl alcohol being added until the 100 ml is used when all the dye will have been absorbed The stain is ready for use within an hour It will be found that a small putty like residue remains but this can be removed and discarded

Of the above methods of preparing Leishman s stain we prefer No 3

For demonstrating the stippling in host cells of all species of *mammalian malaria parasites Leishman s stain is excellent* providing the correct technique is employed (James[1] [2]) Unfortunately however there is considerable variation in the quality of different brands of Leishman stain and therefore samples of *each should be tried until the best is found For staining malaria* parasites Leishman stain should always be prepared in the laboratory and never purchased in solution The dye keeps indefinitely and we are still getting satisfactory results with stain prepared from dry crystals purchased over 20 years ago We are aware that in the tropics Leishman stain is not now extensively used at least not in malaria surveys There are several reasons for this The prepared stain does not keep very long at high temperatures At 27 C it deteriorates in a week or two However

at 16 -17 C it retains its full staining properties up to one year some technical knowledge is required to obtain the best results and it is not as suitable as Giemsa for field surveys After many years of trials and experiments with this stain we believe we have evolved a technique which gives consistent results and that for staining malaria parasites and the stippling in the host cells *in thin films* Leishman is the best of all the Romanowsky modifications Our technique of staining is as follows

On a shelf on the right hand side over the laboratory sink we keep a 5 litre aspirator of distilled water On the same shelf we keep a jar containing two standard tubes of phenol red pH 7 0 and pH 7 2 a cordite tube marked at 5 ml one 50 ml drop bottle containing a filtered saturated solution of lithium carbonate and another containing 0·02 / phenol red Before beginning the day s work we test the pH of the distilled water in the aspirator by running off 5 ml of water and adding 0 5 ml of phenol red If the distilled water is acid a few drops of lithium carbonate are added until the pH registers 7 2 Some is then poured into a 152 mm (6 in ) diameter petri dish exposed to the air and rested on the laboratory bench for 15–20 minutes If at the end of this time the pH is neutral the water is ready for use but if it is acid more lithium carbonate is added to the distilled water in the aspirator and the procedure repeated (As a rough guide when 10 drops of lithium carbonate are added to 5 litres of distilled water the pH is neutral ) Sometimes depending on the amount of $CO_2$ in the atmosphere it may be necessary for the distilled water in the aspirator to have a pH of even 8 0 if it is to have a pH of 7-7 2 after being exposed to the air for 15 minutes which is the optimum time for staining a film If the distilled water used for diluting the stain becomes acid within a few minutes of exposure to the atmosphere on the laboratory bench the films when flushed off at the end of the staining period will be pink Such films seldom show stippling of the host cells and moreover the cytoplasm of the parasite is poorly coloured If on the other hand the water is too alkaline (over 7 6) at the end of the staining period the film will be blue with consequent poor staining of the chromatin The stippling will either be absent or only faintly seen To the naked eye a washed film should be of a faint greyish bronze colour A satisfactory test is to look at the granules of an eosinophile leucocyte If the staining solution is neutral at the time of washing off the film the eosinophile granules will be a pale

greyish colour If the solution has become acid the granules will be bright pink and if it has become *alkaline the granules will* be deep blue For hæmatological work e g differential leucocyte counts workers often prefer a pink coloured film But for optimum demonstration of malaria parasites the red blood corpuscles should be inconspicuous by comparison The parasites are then prominently displayed *morphologically against the greyish* bronze coloured host cells

The importance of the pH of the distilled water for staining with Leishman cannot be over emphasised This is illustrated by Field and Shute[3]

Some workers use buffered distilled water but *we prefer* the water to be adjusted to pH 7 2 with lithium carbonate only But it is not sufficient to adjust the distilled water to pH 7 2 without taking other factors into consideration For example in a laboratory where there are gas fires or bunsen burners exposed distilled water adjusted to pH 7 2 usually becomes acid within a few minutes This also occurs if all the laboratory windows are closed day and night We have tested this on many occasions by staining one film in the laboratory and another in the open air For staining blood films we use a room where there are no coal or gas fires or bunsen burners For routine work where films are not wanted for future reference or class purposes a buffered water at pH 7 2 will save a great deal of detailed work Tablets of buffer salts *sufficient for a litre of water are now available However* it is our experience that films fade much more rapidly when the water is buffered and also the colouring is not quite so vivid

METHOD OF STAINING

*Put 6 or 7 drops of stain on the film* and allow it to act for 15 seconds not more This is sufficient to fix the film and if the stain is left longer than 15 seconds before diluting it especially in hot weather evaporation brings about precipitation and spoils the film Add 12 drops of distilled water (pH 7 2) and thoroughly mix the stain by gently rocking the slide The diluted stain is allowed to act for 15–20 minutes when it is flushed off by a stream of distilled water The flushing should occupy only a few seconds otherwise there is a risk of washing out the *stippling especially* the Maurer s dots of *P falciparum* infections In very hot weather it is advisable to fix the film with one or two drops of methyl alcohol and after it has dried stain as above

For routine examination a slightly acid or slightly alkaline Leishman solution will not prevent malaria parasites from being recognised But species diagnosis is more difficult especially with young trophozoites and in the absence of stippling Another important reason for the staining solution to be neutral *at the end of the staining period* is that it helps to prevent fading For films which are to be kept for class and demonstration purposes it is essential that the staining solution should be neutral at the end of the staining period There are a number of so-called neutral mounting mediums on the market and probably all are satisfactory under optimum conditions Yet when the question as to which is satisfactory and which is unsatisfactory is discussed among a group of workers there is often much disagreement The real reason for the disagreement is we believe often overlooked because it may have nothing to do with the mounting medium but may be due to the film itself If the film is pink (acid) it will fade within a few weeks first the blue of the cytoplasm and then the pink of the chromatin If the film is bluish (alkaline) then the chromatin will fade first followed later by fading of the cytoplasm

It is quite useless to attempt to preserve films of this kind whatever mountant is used On the other hand if the diluted stain is neutral at the end of the staining period and the film is then mounted with a neutral medium it will often keep for years In this laboratory we have blood films which were stained with Leishman and mounted in Euparal vert over 30 years ago (Shute[4]) Some of these specimens are still shown at various laboratory meetings and if fading has occurred it is imperceptible The only precaution we take is to keep the films away from heat and overlong exposure to strong light

In our experience Leishman stain is unsatisfactory for thick films either when the drop of blood is first dehæmoglobinised dried and stained or when Leishman is first diluted and the film stained and dehæmoglobinised at the same time as is usually practised with Giemsa stain Although Leishman is excellent for staining all mammalian malaria parasites in thin films and for staining sporozoites from the salivary glands it serves no other useful purpose It is a poor stain compared with Giemsa for the parasites of avian malaria thick films sections smears of bone marrow post mortem material and exoerythrocytic parasites

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**Giemsa stain**

Unlike Leishman stain Giemsa keeps indefinitely in all climates *It is easily prepared from* Giemsa powder or from the two azures separately

| | |
|---|---|
| (1) Giemsa powder | 3 8 gm |
| Glycerol (pure) | 250 ml |
| Methyl alcohol (certified purity) | 250 ml |
| (2) Azur II eosin | 3 0 gm |
| *Azur II* | 0 8 gm |
| Glycerol (pure) | 250 ml |
| *Methyl alcohol* (certified purity) | 250 ml |

*We prepare the stain by mixing the alcohol* and glycerol and then adding small quantities at a time to the powder in the mortar and grinding until all the colour is absorbed As with Leishman crystals *so it is with Giemsa powder some brands are* better than others In our experience with several brands better results are obtained when the stain is prepared from the two azures instead of from the powder already mixed by the manufacturers but why this is so we do not know It is best not to *filter Giemsa stain for about a week after it is made up A putty* like residue always remains however thoroughly the dye is powdered in the mortar By leaving the mixture for about a week after its preparation before filtering the maximum amount of the dye is absorbed by the glycerol and alcohol The prepared stain *should be kept in a bottle made of hard glass with a close fitting* ground glass stopper and kept away from strong light

After the films have been fixed for half a minute in methyl alcohol or one minute in ethyl alcohol they are ready for staining The dilution of 5–7 ml stain to 100 ml distilled water gives *excellent results Better results are obtained when the water is* neutral rather than slightly alkaline If the water is even very slightly alkaline the red corpuscles stain greyish blue but when this happens it can be corrected by drying the film after staining and then soaking it for a few minutes in normal saline buffered to *neutral*

The stippling of all the four species of human malaria parasites can be demonstrated with Giemsa stain but it is not so vividly displayed compared with the best that Leishman can do *Although we are able consistently to produce* Ziemann s dots in *P malariæ* with Leishman stain *we have seldom succeeded with*

Giemsa even after prolonged staining We find that for thin films staining with Giemsa for 30 minutes gives best results (twice as long as with Leishman)

When films which are not wanted for future reference or for teaching purposes are to be stained in large numbers for routine examination distilled water buffered to pH 7 0 can be used The buffer salts required are the alkaline and acid phosphate potassium dihydrogen phosphates and disodium hydrogen phosphate A buffer solution which gives a pH of 7 2 is

| | |
|---|---|
| Potassium dihydrogen phosphate $KH_2PO_4$ | 0·7 gm |
| Disodium hydrogen phosphate $Na_2HPO$ | 1 0 gm |
| Distilled water | 1000 ml |

The solution should be tested after its preparation and if necessary a little more of one or the other salts added Once a correct pH is obtained the solution remains stable almost indefinitely Tablets of these buffer salts can be purchased either for 100 ml or 1000 ml of water

Thin films stained for from 20–30 minutes give optimum results This procedure stains not only the parasite but also the stippling However if a diagnosis of malaria only is required the parasites are recognisable after staining for only 5 minutes After the staining period is completed the film is flushed with a stream of distilled water This should not be prolonged beyond a few seconds because if the parasites are young trophozoites of *P falciparum* the colouring of Maurer s dots may be washed out If the film is too blue we find it best to dry it and then soak it for a few minutes in normal saline buffered to neutral

We recommend diluting Giemsa stain with distilled water which has a pH of 7 0 buffered if for routine work but for specimens which it is intended to keep for future reference or for class work the distilled water should be brought to a pH of 7 0 with an alkaline reagent such as lithium carbonate

## Giemsa saline for thin films

Pampana[5] was the first to show that Giemsa diluted with saline gave better results than Giemsa diluted with distilled water for thick films but he made no mention of thin films Later Field[6 7] based his rapid method for thick films on Pampana s findings but he too did not mention thin films It was quite by accident that on one occasion we used saline instead of distilled water when

making up Giemsa to stain some thin films When later we examined the films (*P vivax*) we were surprised to see a type of staining quite unlike our routine Giemsa stained specimens The uninfected red cells were semi transparent the leucocytes and the malaria parasites were deeply and beautifully coloured and the stippling was so prominent that the granules could be counted Later we were able to trace the cause of this unusual picture and so we decided to carry out a series of tests with both thick and thin films (Shute[8])

For staining thin films following fixation with methyl alcohol in the usual way Giemsa stain is diluted at the rate of 5–7 ml of stain to 100 ml of saline (0 85 per cent) The parasites are nicely stained after about 30 minutes but best results are obtained when films are stained for one hour There are a number of features about this method of staining all of which appear to be advantageous

(1) The length of time thin films are left to stain seems to be quite immaterial (beyond the minimum of 30 minutes to 1 hour) Beyond that time no changes occur and even when the films are left in the stain for a week the picture is the same as when the films are stained for an hour

(2) The pH is not very important at least providing the water is not acid For example tap water is just as satisfactory as distilled water and films stained with tap water saline and flushed with tap water are as clean and as well stained as those with distilled water The tap water we have tried was the local Epsom main supply By the courtesy of the Epsom Borough Engineer we are able to give the analysis

| | |
|---|---|
| pH | 7 2 |
| Hardness | Total 255 |
| Free carbon dioxide | 18 |
| Total solids | 370 |
| Alkalinity as calcium carbonate | 245 |

A further series of tests were carried out using a local supply from the nearby town of Leatherhead The analysis of this water is

| | |
|---|---|
| pH | 9 1 |
| Hardness | Total 94 |
| Free carbon dioxide | Nil |
| Total solids | 155 |
| Alkalinity as calcium carbonate | 46 |

Films stained with Giemsa and Leishman diluted with this water are quite useless the red cells stain deep blue the parasites are only faintly coloured and the whole film is covered with fine granule deposits Yet when this water is made saline the staining results with Giemsa are quite good particularly when the pH is around 7–7 4

For staining films in bulk this method is excellent In fact we have used the same solution of stain on as many as five occasions *on the same day* staining a hundred films with 100 ml of stain The only disadvantage is that all our efforts to demonstrate Maurer s dots in *P falciparum* have failed even after prolonged staining and when as a control these dots have been beautifully demonstrated by Leishman s stain We have no explanation to offer why this should be so

However it is interesting to remember that Maurer s dots have never as far as we are aware been demonstrated in thick films by any method of staining and we are therefore inclined to doubt whether Maurer s dots of *P falciparum* and Schuffner s dots of *P vivax* are produced by the same mechanism (Shute and Maryon[9])

The reason why saline Giemsa gives so much better staining results than Giemsa diluted with distilled water we do not know It has been suggested by Sir Rickard Christophers that saline is a better protein solvent than distilled water and that it probably removes something from the film which remains after treatment with water

## THICK BLOOD FILMS

### Technique of preparation

There is a tendency to make thick films too thick The drop of blood should not be more than twice or at most three times the size required for a thin film The blood should be spread to cover about three times its original area and this is best done with the corner of another clean slide A simple test to estimate the correct thickness of a film is to place the blood over a piece of newspaper with small print or over the face of a watch The small print or figures round the dial of the watch should just be visible If not then the drop of blood is too thick if seen too clearly it is too thin Some workers advocate a very thick film that has about 20 leucocytes per micro field (2 mm — $\frac{1}{12}$ in —oil immersion lens with a ×5 or 6 ocular) This represents 20 to 30 times the

thickness of a thin film We deplore this advice because (i) scanty parasites especially *P falciparum* rings could easily be hidden beneath a leucocyte (ii) the drop of blood is likely to peel from the slide during staining and (iii) more intensive and prolonged search of each micro field becomes necessary On the other hand with an average of about 10 leucocytes per micro field the above disadvantages are not encountered After the film is prepared it must be put aside to dry in a horizontal position and covered to protect it from dust and flies

It is often stated that as soon as a thick film is dry it is ready for staining This is not our experience and we find that some heat is required to ensure that the drop of blood will adhere to the slide during the staining process If a thick film is to be stained within an hour of taking it we put it on the top of the microscope lamp where the temperature is 50 -60 C and leave it for 7 minutes Too much heat will of course fix the film and prevent the hæmoglobin from dissolving during staining Thick films which have been kept overnight in the incubator at blood heat usually adhere to the slide (Shute[10])

**Technique of staining thick films**

There are four recognised methods of staining thick films all of which we have used in this laboratory There is also a fifth method J S B (Singh and Bhattacharji[11]) which many workers have found useful but which we have not This may be because we have not mastered the technique but since many workers have obtained satisfactory results we are giving the formula

METHOD 1 *Staining thick films with Giemsa stain diluted with distilled water*

The dilution of 5-7 ml of stain to 100 ml of distilled water adjusted to neutral is used The blood films are covered with the stain without previous dehæmoglobinisation and the stain is allowed to act for from 20-30 minutes The films are then gently flushed with distilled water care being taken not to let the stream of water come into direct contact with the blood otherwise there is a risk that the films will be washed from the slide After they have dried following staining it is best to soak them for a few minutes in saline to remove the surface residual stain and the envelopes of the lysed cells These stain greyish blue and so reduce visual contrast and make examination tedious

METHOD 2 *Giemsa stain diluted with saline buffered to* pH 7·2

5–7 ml. stain to 100 ml. saline (0·85 sodium chloride)
Stain for 30 minutes to 1 hour

Pampana[4] was the first to draw attention to the use of an isotonic solution for diluting Giemsa stain for thick films. He pointed out that leucocytes and protozoa were undamaged and that red cells lysed more readily than when distilled water was used.

The technique is the same as that used for ordinary Giemsa staining. The stain is diluted and thoroughly mixed and poured into the vessel containing the slides. We use glass containers with 10 grooves which hold 100 ml. If thin slides are used each groove will hold two and these are placed back to back; thus each container will hold 20 slides.

The films are stained for 30 minutes and the stain is then gently flushed off in the usual way. In *P. vivax* and *P. ovale* the stippling is vividly coloured, but as with all other techniques of staining thick films of *P. falciparum* Maurer's dots are not seen (Shute and Maryon[3].)

METHOD 3 *Field's stain*[15]

This consists of two solutions:

*Solution A*

| | |
|---|---|
| Methylene blue (medicinal) | 0·8 gm. |
| Azure I | 0·5 gm. |
| Disodium hydrogen phosphate (anhydrous) | 5·0 gm. |
| Potassium dihydrogen phosphate | 6·25 gm. |
| Distilled water | 500 ml. |

*Solution B*

| | |
|---|---|
| Eosin | 1·0 gm. |
| Disodium hydrogen phosphate (anhydrous) | 5·0 gm. |
| Potassium dihydrogen phosphate | 6·25 gm. |
| Distilled water | 500 ml. |

The phosphate salts are dissolved and then the stain is added. Leave for 24 hours and then filter.

If the anhydrous salt is not available, sodium phosphate cryst. B.P. ($Na_2HPO_4 \cdot 12H_2O$) 12·6 grammes can be used.

**Method of staining**

On the bench by the sink place 3 wide mouth staining jars *101 mm × 38 mm (4 in × 1½ in)*

| | |
|---|---|
| Number 1 | Solution A |
| Number 2 | Buffer (without stain) |
| Number 3 | Solution B |

(*a*) Dip the slide for one second in Solution A
(*b*) Wash off the stain from the back of the slide with distilled water
(*c*) Dip the slide in the buffer solution until most of the excess blue colouring has left the film
(*d*) Dip the slide for one second in Solution B
(*e*) Wash gently for a few seconds with distilled water to remove excess eosin
(*f*) Place slide film downwards in rack to dry

*The chief advantage of this stain over ordinary Giemsa diluted* with distilled water is that the background of the film is quite clean the parasites and leucocytes are sharply defined and the leucocytes are not distorted A disadvantage is that by this technique films cannot be stained in bulk and though useful for the clinical laboratory it is not suitable for field survey work when large numbers of films have to be stained However as a method of staining individual thick films it is a great improvement on the ordinary Giemsa method

Because thick films cannot be stained in bulk by Field s method for field surveys it is better to stain with Giemsa diluted with saline With the latter method the background is quite clean and the parasites and leucocytes are more vividly coloured than they are with Field s stain

METHOD 4 *Field s method and counterstaining with Leishman* (Shute[14])

It is only for convenience that we are describing this as a separate method the only difference from Method 3 being that after the film has dried it is again stained with Leishman exactly as though it were a thin film Very beautiful results are obtained and for special purposes the extra time and labour required are well worth while The background of the film remains quite clean the parasites are intensely coloured and in *P vivax* and

*P ovale* the stippling is stained as vividly as in an ordinary thin film All our attempts to demonstrate Maurer s dots by this technique have failed We are therefore inclined to the belief that it is not possible to demonstrate Maurer s dots in other than thin films and only in these when the trophozoite has been attached to the cell for some time probably over 12 hours

METHOD 5 *The J.S B stain*[11]

There are two solutions

*Solution 1*

| | |
|---|---|
| Medicinal methylene blue | 0 1 gm |
| Potassium dichromate | 0 1 gm |
| Sulphuric acid (1 by volume) | 0 6 ml |
| Potassium hydroxide (1 ) | . 0 ml |
| Water | 100 ml |

*Solution 2*

| | |
|---|---|
| Eosin (water soluble) | 1 0 gm |
| Tap water | 500 ml |

**Method of staining thick films**

1 Immerse slide in solution 1 for 10 seconds

2 Wash in jar containing acidulated water (pH 6 2–6 6) for 2 seconds

3 Stain with solution 2 for one second

4 Wash in same jar (2) for 5 seconds

5 Immerse in solution 1 again for 10 seconds

6 Wash as above for 2 seconds or till smear gives a pink background (2)

7 Dry

**J S B method of combined staining**

1 Fix the thin smear by dipping that part of the slide in a jar containing methyl alcohol for a second or two

2 Dry thoroughly preferably by waving the slide in the air

3 Immerse the whole slide in solution 1 for 30 seconds

4 Wash in a jar containing acidulated tap water (pH 6 2–6 6) With tap water in Delhi (pH 7 6 indicator bromo thymol blue) approximately 50 mg of sodium dihydrogen phosphate or 5 drops of 5 acetic or citric acid solutions for each 100 ml of water are necessary

5 Stain with solution 2 for one second
6 Wash in same jar (4) for 4 seconds
7 Immerse in solution 1 again for 30 seconds (3)
8 Wash as above for 10 seconds or until the smear gives a pink background (4)
9 Dry

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## SECTION 2

## MISCELLANEOUS LABORATORY PROCEDURES

### PREPARING BLOOD FILMS FOR DEMONSTRATING EXFLAGELLATING MALE GAMETOCYTES

MATERIALS REQUIRED

1 Nests of petri dishes 88 mm (3½ in ) diameter
2 Filter paper
3 Triangular pieces of 6 3 mm (¼ in ) glass rod or tubing
4 Spirit lamp
5 Glass slides

Prepare five or six petri dishes by inserting a layer of filter paper accurately cut to fit in the lid and in the bottom of each (James[1]) Lay a triangular piece of glass rod or tubing in each dish and moisten the filter paper with as much *hot* water as it will absorb but not more The filter paper should be wetted to saturation at least one hour before the dishes are to be us d Take the dishes and the slides to the bed of the patient accompanied by an assistant who will hold a separate dish ready for each blood film as it is made Clean and prick the finger make a film slightly thicker than an ordinary thin film and at once breathe on it and quickly lay it on the glass rod in the dish from which the assistant has momentarily removed the lid The lid is quickly replaced and more specimens are prepared in the same way The dishes must be kept quite level and taken to the laboratory without delay (unless the temperature in the room where the specimens are prepared is 23 C or higher) If it is required to see the early changes in the process of exflagellation remove a slide from a dish at 5 minute intervals At 25 C exflagellation is completed in 12–15 minutes for *P vivax* and *P ovale* and in from 15–30 minutes for *P falciparum*

After removing the slide from the petri dish quickly wipe the under side of the slide on the back of the hand This enabl s one to make sure that the blood film has not dried If it is still moist pass the slide once only through the flame of a spirit lamp to dry the film quickly For special cytological studies instead of passing

the film through the flame of a spirit lamp expose it to osmic acid vapour for a few seconds If the film is dry when taken out of the petri dish this is either because the atmosphere in the dish is not sufficiently moist or because the film dried before it was put into the dish Such films should be discarded

Atmospheric temperature is very important Below 15 C exflagellation does not occur at 16 -20 C it may take as long as half an hour whereas at 25 C it occurs in 12-15 minutes after 20 minutes free flagella and often fertilisation of the female parasite can be seen

REFERENCE

1 JAMES S P (1934) *Trans R Soc trop Med Hyg* 28 104

PREPARING SPECIMENS OF OOKINETES OR VERMICULES

Feed some anopheles on a patient whose blood contains numerous gametocytes the males having been tested for ripeness (exflagellation) Select and transfer to a very small cage mosquitoes which have gorged themselves with blood and keep them in a room where the temperature is 24 -27 C and the humidity is relatively high (80 per cent) At intervals up to 24 hours remove one or two mosquitoes and proceed as follows

Anæsthetise the mosquito and remove its legs and wings (stunning the mosquito in the test tube will nearly always rupture the stomach and spoil the specimen because the blood will stick to the inside of the test tube)

Dissect the mid gut in saline or Locke s fluid by nicking the chitin on both sides of the 5th or 6th abdominal segment so as to draw out the mid gut containing the blood clot without tearing the wall of the stomach Cut away the malpighian tubules lift the clot on to the point of a dissecting needle and transfer it to a drop of Locke s fluid previously placed on another clean slide about one inch from one end of it With the aid of the dissectin microscope against a white background tear the clot so that the blood becomes mixed with the Locke s fluid The released blood should be confined to as small a space as possible so as to prevent any part of it from drying When the blood and the fluid are thoroughly mixed make thin films on clean slides in the same way as if the material were a drop of finger blood After the films have dried stain them in the same way as a blood film with either Giemsa or Leishman

Search for ookinetes with the 2 mm ($\frac{1}{12}$ in) oil immersion lens The amount of saline used should be about the same volume as the blood clot Some dilution but not too much is necessary because the blood being semi digested and coagulated contains much debris and if undiluted examination would be difficult (James[1])

It should be noted that ookinetes will form in the gut of any blood sucking arthropod The very large culicine *Theobaldia annulata* takes at least twice the quantity of blood that is imbibed by most anophelines and we have often prepared as many as 16 blood smears from the diluted stomach content of a *T annulata* which had fed on a gametocyte carrier 24 hours earlier for demonstration purposes

REFERENCE

1 JAMES S P (1934) *Trans R Soc trop Med Hyg* 28 104

## TECHNIQUE FOR DISSECTING THE SALIVARY GLANDS AND STOMACH OF A MOSQUITO

MATERIALS REQUIRED

1 Ordinary glass slides 75 mm × 25 mm (3 in × 1 in)
2 19 mm ($\frac{3}{4}$ in) No 1 square cover slips
3 Two bayonet shaped dissecting needles
4 Dissecting microscope
5 Ordinary microscope with mechanical stage removed
6 Test tubes 127 mm × 12 7 mm (5 in × ½ in)
7 Watch glass containing normal saline
8 Chloroform or ether

Transfer the mosquito to be dissected to a narrow test tube and when the insect is at the bottom rap the end of the tube sharply against the palm of the hand five or six times This is sufficient to stun the mosquito which is then removed to a glass slide Holding the insect by one wing remove the legs *one at a time* and afterwards pull off one wing Return the mosquito to the slide and with a dissecting needle cut off the remaining wing The insect is now devoid of wings and legs and ready for dissection (Shute[1])

Place the insect in the centre of a dry slide with the anterior end pointing to the right and with a needle in the right hand cut off the head sharply and cleanly Place a drop of saline (the size

of a pin s head) quite close to but not touching the anterior end of the thorax The point of the dissecting needle held in the left hand is gently but firmly planted on the thorax *just below th* parts where the glands lie With a needle in the right hand bring gentle pressure to bear on the thorax a little above the left hand needle Very little pressure is needed to express the glands from the thorax As soon as this occurs touch the bead of saline with

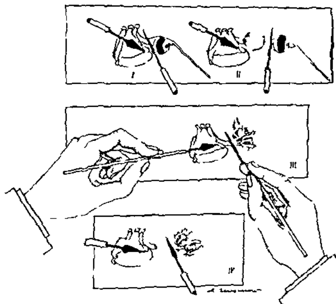



Fig 1 The dissection of a mosquito

the point of the right hand needle and bring it into contact with the glands Next remove the mosquito and transfer it to the watch glass containing the saline to await the dissection of the mid gut

Transfer the slide containing the salivary glands to the platform of the microscope and bring the specimen into focus with the low power (16 mm —$\frac{5}{8}$ in —lens) Reduced light should always be used and the glands should be in the centre of the slide When the glands are in focus and in the centre of the microscopic field drop a 19 mm ($\frac{3}{4}$ in ) square coverslip on to them at an angle so that one corner of the coverslip is over the glands and the opposite

corner protruding beyond the slide The coverslip should be dropped sufficiently heavily on to the glands to rupture the cells without displacing them The object of applying the coverslip at an angle is to prevent the saline from becoming too widely distributed and so make the examination easy and quick as the glands and their contents are concentrated in a very small area Focus the specimen with the 4 mm (⅙ in ) lens and examine for *sporozoites*

Instead of stunning the mosquito the insect can be anæsthetised with ether or chloroform by dropping two or three drops on to the plug of cotton wool covering the mouth of the test tube This is preferable if the mosquito has blood in the stomach or contains ripe eggs Care should be taken not to use too much chloroform or ether otherwise some of it may wet the mosquito and cause partial fixation and so make dissection difficult

### To dissect the mid gut

If the glands have been dissected with care the remainder of the insect will be undamaged Lift it from the watch glass containing the saline on to the centre of a glass slide The insect should be lying *on* a drop of saline with the thorax pointing to the left Steady the thorax on the slide by transfixing it with the point of the left hand needle With the right hand needle nick the chitin above and below on either side of the 6th or 7th abdominal segment Gently pull the detached segments with the right hand needle until the mid gut and the attached malpighian tubules come into view Sever the alimentary canal sufficiently far forward to bring away a portion of the fore gut If the correct quantity of saline has been used the malpighian tubules will tend to float backwards leaving the whole of the mid gut clear Anchor the gut by placing the point of the left hand needle on the fore gut and with the right hand needle cut through the alimentary canal at the junction of the mid and hind gut severing the base of the malpighian tubules at the same time Remove the hind gut and malpighian tubules so that only the mid gut (with the attached tag of œsophagus) remains on the slide Let one edge of the coverslip rest on the slide but not in contact with the stomach and the opposite edge rest on the point of the dissecting needle which is then gradually lowered till the coverslip comes to rest on the stomach If the stomach is lying on too small a drop of saline the weight of the coverslip will rupture the stomach cells

and this will spoil the preparation and make examination for oocysts difficult On the other hand if too much saline is used the stomach will not flatten sufficiently in this case some of the excess saline can be removed by holding a strip of filter paper against the edge of the coverslip

**To examine for oocysts**

Place the slide containing the specimen on the platform of the microscope and examine with the 16 mm ($\frac{2}{3}$ in ) lens with reduced light (it is best not to use the mechanical stage but to manipulate the specimen with the index finger and thumb of the left hand) If large oocysts are present they are easily seen with the low power of the microscope Failing this examine with the 4 mm ($\frac{1}{6}$ in ) lens and if no oocysts are seen examine with the 2 mm ($\frac{1}{12}$ in ) oil immersion lens For this it may be necessary to use the mechanical stage of the microscope

Mosquitoes which are infected and kept at 25 C show oocysts under the following magnifications

| | |
|---|---|
| 3–4 days | 2 mm ($\frac{1}{12}$ in ) lens × No 6 eyepiece |
| 5–7 days | 4 mm ($\frac{1}{6}$ in ) lens × No 6 eyepiece |
| 8–11 days | 16 mm ($\frac{2}{3}$ in ) lens × No 6 eyepiece |

*It is often easier to detect the very young oocysts 3–4 days old* than those which are about half grown In the very young oocysts the pigment is concentrated within a very small sphere whereas in those which are half grown it is distributed over a much larger area of the cyst and much of it is obscured by the dividing nuclei There should be no difficulty in detecting large oocysts because their sporoblast or sporozoite contents are prominent Reduced light should be used throughout

When large numbers of mosquitoes are to be dissected it is a good plan to have on the laboratory bench a large sheet of white paper and a piece of black paper about 152 mm (6 in ) in diameter covered by a piece of plate glass For removing the legs and wings of a mosquito a white background is helpful but for seeing clearly the dissected tissue (stomach and glands) a black background is necessary There should also be a black platform on the dissecting microscope for the same reason As regards the type of dissecting microscope the simple model with a ×10 eyepiece and arm rests *appears to us to be quite adequate it leaves plenty* of room for manipulating the needles and the magnification is sufficient for the tissues to be seen clearly With this type of microscope

we have frequently dissected 200 mosquitoes in a day without undue fatigue We cannot see any advantage in using binocular microscopes for these dissections and after extensive trials we consider that they slow up the work and increase fatigue However the simple monocular microscope mentioned above is only 152-203 mm (6-8 in ) high and when large numbers of mosquitoes are to be dissected it is advisable to rest the instrument on a block of wood or other material about 101 mm (4 in ) thick to prevent having to stretch the neck at an awkward angle for long periods It is also an advantage when carrying out many dissections to have two small petri dishes each containing a wet plug of cotton wool one for the right hand needle and one for the left Following a dissection the head and proboscis of the mosquito usually adhere to the right hand needle and the exoskeleton of the abdomen to the left hand needle The wet plugs of cotton wool enable one quickly to wipe the needles

The dissecting needles

With practice almost any type of needle can be used but one *which is well designed facilitates speedy and clean dissections* The pattern we prefer is made of stainless steel bayonet shaped with a sharp cutting edge and a rubber covered handl * This is important because if the blade becomes rusty the dissected tissue will stick to it The edge of the needle is sharp making it easy to sever the tissue while the rubber holder ensures a firm grip

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## TO MAKE PERMANENT AND STAINED PREPARATIONS OF SPOROZOITES AND INFECTED MID GUTS

MATERIALS REQUIRED (FOR SPOROZOITES)

1 Leishman or Giemsa stain
2 Euparal
3 No 1 coverslips 19 mm (¾ in ) diameter

Before staining make a circle surrounding the specimen with a blue grease pencil on the reverse side of the slide This makes it easy to locate the specimen when the time comes to examine it

* This type of dissecting needle designed to our specification is manufactured by Baird & Tatlock Ltd Chadwell Heath Essex and is catalogued 'The Shute Dissectin Needle

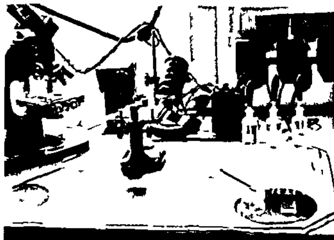


Fig 2 Equipment and arrangement for mass dissections of mosquitoes



Fig 3 Pair of dissecting needles and petri dish containing a wad of wet cotton wool



Fig 4

Sporozoites stain equally well with either Leishman or Giemsa The technique is the same as for blood films but it is important that the pH of the distilled water be adjusted to neutral or pH 7 1 7 2 otherwise the nucleus and the cytoplasm will not be prominently displayed

If the specimen is worth keeping mount it in Euparal vert with a 19 mm (¾ in ) diameter No 1 coverslip

MATERIALS REQUIRED (FOR MID GUTS)

1 Saline
2 Bles fixative 90 ml 70 / alcohol
   7 ml formalin
   3 ml acetic acid (glacial)
3 MAYER s hæmalum (Pantin[1] 1948) in 1000 ml distilled water dissolve 1 gramme hæmatoxylin
   0 2 gramme sodium iodate
   50 grammes potassium alum
   Shake frequently until the solution is blue violet (some hours) add 50 grammes chloral hydrate
   1 gramme citric acid crystals
   The solution now turns red violet Keep in a hard glass bottle Mayer s hæmalum is a progressive self differentiating stain nuclei blue cytoplasm pink
4 Carbol xylol 30 / phenol dissolved in xylol
5 Canada balsam
6 Coverslips No 1 19 mm (¾ in ) diameter

Dissect the gut in normal saline in the centre of a clean slide as already described Then proceed as follows

See that the gut is floating on the saline if it is sticking to the slide it will rupture when the coverslip is applied With a dissecting needle gently lower a 19 mm (¾ in ) diameter coverslip on to the specimen to flatten it if it is dropped on heavily the gut will rupture and be spoilt With practice the right quantity of saline required to flatten the tissue will quickly be learnt If there is too much saline the gut will not be evenly flattened and if there is not enough it will rupture Without touching the coverslip place one drop of Bles fixative on the slide so that it will run under the coverslip mix with the saline and so begin the process of fixation Leave for 2 or 3 minutes and then add another drop of fixative After 5 minutes the specimen will be partially fixed and it is then

transferred to a petri dish containing fresh Bles to complete fixation. Care must be taken not to disturb the coverslip. Leave for half an hour or longer and then remove the coverslip with the aid of dissecting needles. The petri dish containing the specimen should be resting on a black surface so that the gut can be seen clearly. Hold the needle in the left hand so that it is just touching the edge of the coverslip. Hold the right hand needle at an angle of 45° and very gently insert its point between the coverslip and the slide. The needle in the left hand must prevent the coverslip from sliding while the right hand needle gently raises the coverslip. In most cases the gut will be found adhering to the latter. If the gut adheres to the slide the coverslip can be discarded. If it adheres to the coverslip continue lifting the latter by keeping the left hand needle steady until the coverslip turns over with the tissue uppermost. When fixation is complete the gut will become opaque. Transfer the specimen to 50% alcohol for 15–20 minutes and then for about the same time immerse it in slightly alkaline distilled water.

**To stain the gut**

In our experience the most satisfactory stain is Mayer's hæmalum. If the stain is allowed to act for about half a minute or less the oocysts are nicely stained and stand out prominently against the surrounding tissue cells. Most other hæmatoxylins we have tried have not given satisfactory results because the depth of staining of both tissue and oocysts has been about equal. With Mayer's hæmalum and also with Weigert's hæmatoxylin there is better differentiation between oocysts and the surrounding tissue cells. We prefer Mayer's hæmalum to Weigert's hæmatoxylin because it keeps better and it is not necessary to prepare two solutions on every occasion.

After staining the specimen is "blued" in the usual manner and taken through graded alcohols, a few minutes in each being sufficient. To ensure complete dehydration the specimen is placed in carbol xylol for some minutes and then given two or three changes of xylol; every trace of carbol xylol must be removed otherwise fading soon occurs.

If the gut is on the slide it is mounted in Canada balsam in the usual way. If, however, it is on the coverslip it should be mounted as follows.

A drop of balsam is placed in the centre of a clean slide and the

coverslip applied but with the gut on the upper surface Then a further drop of balsam is placed on the gut and another clean coverslip applied The reason for this is that the oocysts on the upper surface of the gut are stained but not those on the under surface

## REFERENCE

1 PANTIN C F A (1948) Notes on microscopical technique for zoolo ists Univers ty Press Cambrid_e

## SECTIONING AND STAINING SECTIONS OF MOSQUITOES

MATERIALS AND SOLUTIONS REQUIRED (PANTIN[1])

Fixatives
Industrial methylated spirit 74 O P
Benzene
Methyl salicylate
Methyl benzoate + 1 / celloidin
Methyl benzoate + 3 celloidin



Fig 5 Longitud nal sect on of infected mosquito The gut is studded with oocysts and some are free in the lumen

Fig 6 Longitudinal section of a maxillary palp of *A stephensi* showing masses of *P vivax* sporozoites in the lumen



Fig 7 Transverse section of mid gut of *A stephensi* showing large numbers of mature *P vivax* oocysts Specimen shows the remains of a blood meal taken 36 hours before dissection

**To section whole mosquitoes**

Anæsthetise the insect in a 127 mm × 12 7 mm (5 in × 1 in ) test tube and remove its wings and legs Hold the mosquito by one wing and remove the legs one at a time and then one wing *Lay the insect on a slide and detach the remaining wing with a* dissecting needle (there is no need to puncture the mosquito to enable the fixative and other fluids to penetrate)

FIXATIVES

We find that the two following fixatives give satisfactory results and do not interfere with subsequent staining or cause brittleness

1 *Ca noy s fixative*

60 ml absolute al ohol (industrial methylated spir t 74 O P )
30 ml chloroform
10 ml acetic acid (glacial)
Fix for 1 ? hours not longer

2 *Bles f xat ve*

90 ml 0 alcohol
7 ml formal n
3 ml acetic acid (glacial)
Fix for 1? 24 hours

*A* Transfer to 90 alcohol (two changes) and leave for 12 hours or overnight

*B* Absolute alcohol or 74 *O P* Two chan es at one hourly intervals

C Methyl benzoate + 1 celloidin (12–24 hours) (Pantin[1])

If necessary objects can be kept in *C* indefinitely A camel hair brush is useful for transferring the specimen from one solution to another

**To embed the specimen**

Dip a square coverslip in melted wax and after it has cooled transfer it to a petri dish and add a large drop of 3 celloidin in methyl benzoate solution on the centre of the waxed coverslip The celloidin blob should be large enough for the tissue to rest quite flat and well within the celloidin With a small camel hair brush carefully lift the mosquito from the celloidin in methyl benzoate solution and transfer it to the centre of the drop Again with a small camel hair brush orientate the mosquito so that it is in the position required for sectioning and then flood the coverslip

with benzene to gel the celloidin This usually takes from 1–2 hours After the wax on the coverslip has dissolved the specimen will leave the coverslip After a further change in benzene embed the specimen in paraffin wax (56 –60°C) two changes at one hourly intervals Block out in the usual way If when cutting the sections they fail to leave the knife as a ribbon remove the block and dip it in overheated wax at a lower temperature e g 45 C But this is not usually necessary when the temperature of the room is 21 –24 C

**To prepare sections of the dissected gut**

Carnoy or Bles are quite satisfactory for fixation If the mosquito had a blood meal 36–48 hours previously and has not completely digested it the gut keeps its shape and the dark colour of the blood makes it easy to follow the specimen through all the various solutions and also for embedding and sectioning

If Carnoy is used 30 minutes is sufficient If Bles is used *2–4 hours are necessary The procedure is then the same as for* sectioning whole mosquitoes However considerable stretching of the tissue occurs during the act of dissecting the gut When therefore a gut is to be sectioned it should after dissection be floated in saline for about 5 minutes to allow the tissue to contract back to normal It can then be transferred to the chosen fixative

**Demonstration of ookinetes in transit**

*To follow up the various developmental processes specimens* should be prepared at 4 hourly intervals beginning 12 hours after the infective blood meal and continuing for 48 hours For this study we prefer transverse sections of the whole mosquito since owing to the stretching of the wall after a blood meal there is always a risk of damage to the gut during the process of dissection with consequent loss of part of the clot

Some of the ookinetes formed close to the gut wall will pass *through or between the cells and become* oocysts in less than 24 hours though still showing a single nucleus Others may be seen at right angles to the axis of the gut in the act of penetrating the wall Marked displacement of the endothelial cells may be seen at the point of penetration

Ookinetes *formed in the central zone of the clot do not reach* and penetrate the wall for several hours after those which happen to be near it at the time of formation Many of these become

phagocytosed before reaching the wall Ookinetes may be found in the lumen of the gut as late as 48 hours after the infective feed and on many occasions we have seen them in the fæces of mosquitoes especially in specimens collected 36 to 48 hours after the infective feed These findings may in part explain why when more than one species of Anopheles are fed on the same human host the number of oocysts seen in the smaller species often exceeds that found in the larger

We prefer Carnoy s fixative whether for the whole mosquito or the dissected gut Sections may be stained either with hæmatoxylin or with Giemsa For demonstration of the nuclei in very young oocysts the modification of Giemsa recommended by Shortt and Cooper[2] for staining exo erythrocytic parasites is to be preferred

### Salivary glands

Sections of the salivary glands can be prepared in the same way as for the dissected gut However these are best displayed by cutting longitudinal sections of the whole mosquito

An important advantage of this technique is that complete dehydration and clearing is obtained without the use of xylol also the time required for the tissue to remain in absolute alcohol is quite short by comparison with other techniques and so there is no risk of the tissue becoming brittle

### Staining

For staining sections of mosquitoes with malarial oocysts well matured Ehrlich s hæmatoxylin is excellent

1 Stain with undiluted Ehrlich s hæmatoxylin for 30 minutes (or longer)

2 Wash in water (a few minutes)

3 Soak in 1 HCl in 70 alcohol (a few seconds)

4 Wash in running water for 5–10 minutes and then leave for a further half hour in water which has been made alkaline by adding a few drops of a saturated solution of lithium carbonate

5 Upgrade in alcohol 50 75 and 90 a few minutes in each

6 Counterstain in 0 01 alcoholic erythrosine 15 minutes or longer

7 Absolute alcohol (industrial spirit 74 O P ) for 15 minutes

8 Carbol xylol for 15 minutes

9 Xylol (two changes until clear) All traces of phenol must be washed out by xylol otherwise rapid fading occurs
10 Mount in Canada balsam

The very thin coating of celloidin which covers the sections seems not to interfere with the staining If however it is considered advisable to dissolve the celloidin before staining this can be accomplished by passing the slide through a solution of equal parts of ether and alcohol This of course should be done *after* passing the specimen through absolute alcohol and after th wax has been dissolved with xylol Neither alcohol alone nor ether alone dissolves the gelled celloidin it must be a mixture of the two

To demonstrate sporozoites in sections stain with Giemsa the technique of Shortt and Cooper for staining exo erythrocytic parasites in the liver gives good results If it is intended to stain sections with Giemsa the fixative must not contain formalin Although tissues treated with fixatives containing formalin do not stain satisfactorily with any of the Romanowsky formulations those fixed with Carnoy stain well with all the hæmatoxylins we have tried and also with the Romanowsky formulations

It sometimes happens that when a mosquito is put into fixative it fails to sink to the bottom of the fluid Usually the reason is that there is an air bubble at the top of the underneath part of the thorax Unless this is dispersed the insect should be discarded because the fixative and clearing solutions will not penetrate

REFERENCES

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## PREPARING SPECIMENS OF SPOROZOITES FROM RUPTURING OOCYSTS

Although the salivary glands of a mosquito may be heavily infected specimens prepared from them seldom show more than a few sporozoites per micro field As the result of dissecting and crushing the glands beneath a coverslip the fluid containing sporozoites is usually distributed over a fairly large area of the slide If however the specimen is dissected dry and a small bead of saline is immediately placed on the glands which are then crushed with

only the corner of the coverslip the sporozoites are concentrated in a very small area By this method however only one specimen is obtained from each set of glands If numbers of specimens are required these can be prepared from mature and rupturing oocysts The technique is as follows

Feed a batch of mosquitoes on a good gametocyte carrier and incubate them at about 25 -26 C On the 4th and 5th day after feeding dissect a few and examine the stomachs for oocysts If the number of oocysts per gut averages less than about 10 the batch should be discarded as unsuitable If the oocysts are numerous incubate the mosquitoes at 25 C until the 7th day after feeding and then remove them to a room where the tempera ture is 16 C During this period they should be fed on 10 glucose soaked in cotton wool Reducing the temperature to 16 C slows down but does not stop the development of oocysts It must be remembered that the number of oocysts per gut varies enormously within a given batch of mosquitoes even when they have all fed at the same time on a patient in whose blood gameto cytes were very numerous The following figures illustrate this point

Twenty eight *A maculipennis atroparvus* were fed on a single occasion on a patient whose blood contained 850 exflagellating male and 1 920 female *P vivax* parasites

*Re ult of d ssections*

| Ser al number | Oocysts | Serial number | Oocysts |
|---|---|---|---|
| 1 2 3 | 0 | 1 | 9[illegible] |
| 4 5 | 9 | 18 | 113 |
| 6 | 15 | 19 | 129 |
| 7 | 26 | 0 | 130 |
| 8 9 | 31 | 21 | 14[illegible] |
| 10 | 36 | [illegible] | 15 |
| 11 | 65 | 23 | 184 |
| 12 | 68 | 4 | 2[illegible]9 |
| 13 | 77 | 25 | 238 |
| 14 | 86 | [illegible]6 | 3 6 |
| 15 | 88 | 7 | 4[illegible]5 |
| 16 | 90 | 8 | 738 |

On the 10th-15th day after infecting the mosquitoes dissect one or two and examine the gut with the 16 mm (⅔ in ) lens without

applying a coverslip to ascertain approximately the number of oocysts If they are sufficiently numerous again examine the preparation this time with a 4 mm ($\frac{1}{6}$ in) lens still without applying a coverslip If some oocysts are ripe and rupturing free sporozoites will be seen moving about in the fluid Having obtained a specimen in this stage of development quickly prepare a dozen slides as follows

In the centre of each slide put a bead of saline no larger than the head of a pin With the aid of the dissecting needles manœuvre the mid gut on to the tip of one of them and gently pass it 3 or 4 times through the bead of saline In most cases it will be found that the mid gut tissue will adhere to the point of the needle and the procedure is then repeated until all the dozen slides have been prepared If the gut leaves the point of the needle while preparing one specimen it can easily be picked up again It is best to do this under the dissecting microscope against a dark background It is also a good plan to add a small bead of fresh blood to about *5 ml of the saline which is used in the dissections* The red cells make it easy to focus the specimen whereas the sporozoites being refractile make focusing difficult even with reduced light After the saline on the slide has dried and before applying the stain encircle the specimen with a grease pencil or diamond marker on the reverse side of the slide Unless the drop of blood is added the specimen being semi transparent is not clearly visible even after staining From a few mosquitoes with mature and rupturing oocysts many specimens of sporozoites can be prepared providing the oocysts rupture without artificial pressure The sporozoites are as perfectly formed as they are when prepared from the salivary glands They are of course much more numerous and as we have shown (Shute[1]) they are infective

It may be thought that a more simple method would be to crush the stomach beneath a coverslip and then to make several specimens from the emulsion But oocysts do not all develop at the same rate so that crushing the stomach in this way would produce an abnormal picture Some immature oocysts would be ruptured and the specimen would show a number of stumpy and immature sporozoites

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### SPECIES DIAGNOSIS OF HUMAN MALARIAL OOCYSTS BY THE ARRANGEMENT AND DEGREE OF COARSENESS OF THE PIGMENT

It is generally agreed that there are no discernible morphological differences between the sporozoites of the four species of human Plasmodia This however is not the case with oocysts

The number of grains of pigment in an oocyst varies from species to species as also do the colour of the pigment and the pattern the pigment forms in the cyst (see Figs 8–11) The pattern varies from day to day during the growth of the oocysts an observation which shows the great importance of a knowledge of this changing character in species diagnosis In addition to being able to identify the species concerned one can also estimate from the character of the pigment the number of days before the oocysts would have ruptured taking into consideration of course the prevailing atmospheric temperatures That temperature plays a vital part in the time required for the parasite to complete its development in the insect host is too well known to need further emphasis It is however not so fully realised how much variation occurs in the time factor between different species at a given temperature At a constant temperature of 25 C the parasite of *P vivax* completes its cycle in 9 days *P falciparum* in 11–12 days *P ovale* in 14–15 days and *P malariae* in 15–21 days It is interesting to note that the two less common species take the longest time to complete their development in the mosquito

There is no evidence to suggest that there are any differences in the arrangement and degree of coarseness of the pigment of a particular species of parasite in different species of Anopheles The descriptions given by us are from studies carried out with infections seen in *Anopheles maculipennis atroparvus* and *A stephensi* (Shute and Maryon[1])

It should be remembered that although quinine does not sterilise the gametocytes of *P falciparum* some of the synthetic drugs do so either partially or completely Mepacrine may cause the gametocytes of *P falciparum* to lose their pigment without sterilising them Oocysts produced from these pigmentless gametocytes do not of course conform to pattern Therefore in areas where malaria control includes mass drug therapy other than quinine atypical oocysts may occasionally be seen

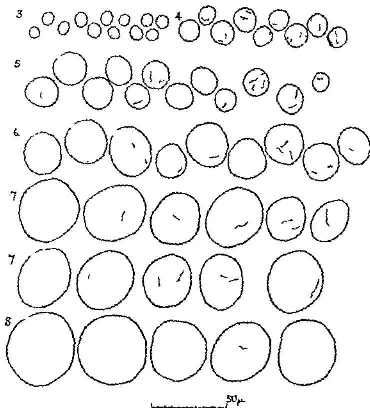



Fig. 8. The pigment is greenish brown but some of the granules are darker than others and may be dark brown. The granules are uneven in size and shape but are mostly in short rods. The number of granules is between 50 and 100. The completion of the cycle takes only 9 days at a constant temperature of 25° C. The pigment is fine and because the grains are so numerous does not form any pattern in very young oocysts (3 days). When the cyst has increased in size and is 4 and 5 days old there is a tendency to a pattern with the pigment in chain formation often in three lines and resembling Prince of Wales feathers. By the sixth day of growth multiple division of the nuclei will have obscured much of the pigment and by the seventh day only a few grains can be seen. On the eighth day it is extremely difficult to see any pigment.

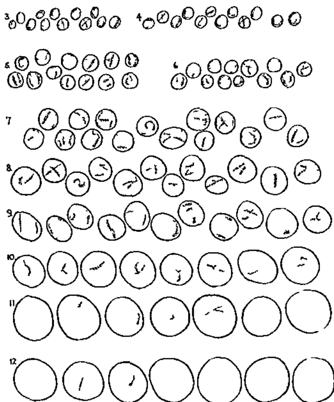



Fig 9 The p gment is very dark brown and the granules thou h not so large as n *P f lcipa um* are larger than those of *P r rax* They compare fa ourably in size and colour with *P mala i e* There are between 50 and 60 gra ns in an oocyst The completion of the cycle takes 15 days at 5 C A peculiar f ature of the p gment is its tendency to form chains wh ch cross each other at right angl s in the centre of the cyst Th s is seldom if ever seen in any other species and it is a good dia nost c character It occurs in oocysts from the fourth day of growth and is most pla nly seen on the s xth to e ghth day From the eleventh day onwards much of the p gment is obscured but some can usually be seen

*P falciparum*

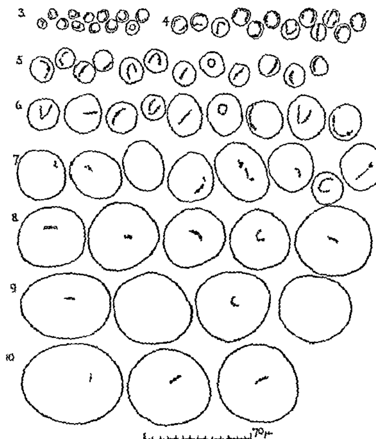
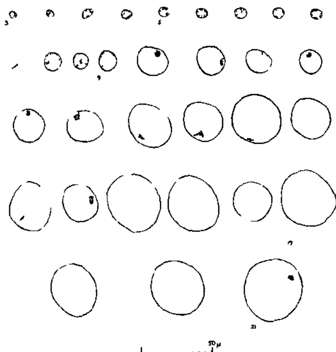



Fig 10 The pigment is almost bla k and the granules are relatively large The number of granules varies between 10 and 20 The completion of the cycle takes 10 days at 25 C *The pigment is very coarse and forms a pattern* This is usually a double semicircle around the periphery of the cyst but it may be a small circle in the centre of the cyst or even a double straight chain This is the usual picture in oocysts aged 3-7 days By the eighth day much of the pigment becomes obscured but a few grains can usually be seen Even on the ninth day *when there is sporoblast formation odd grains of pigment are usually seen*




NUMBERS DENOTE AGE IN DAYS

FIG 11 The pigment is very dark brown and the granules are larger than those of *P vivax* but smaller than those of *P falciparum* There are seldom more than 30 granules and these vary considerably in size During the first 7 8 days of growth the pigment is distributed over the cyst but from about the ninth day onwards it is clumped and is invariably situated in a mass at the periphery of the oocyst Even in young oocysts a degree of clumping may be seen and give the appearance of very large lumps of pigment which are in fact accumulated granules The clumping of the pigment in a small area at the periphery of the cyst is of much diagnostic value

TABLE 1

| Species | Colour of pigment and degree of coarseness | Day by day arrangement of the pigment | Number of grains of pigment | Number of days for completion of cycle | Measurement of oocysts in μ (smallest and largest) |
|---|---|---|---|---|---|
| *P. vivax* | Greenish brown<br>Fine | 3 days Without pattern<br>4–5 days In grains often arranged in Prince of Wales feather formation<br>6–7 days Much of pigment obscured by dividing nuclei<br>8–9 days Seldom seen | 50–100 | 9 | 10–46 |
| *P. ovale* | Dark brown to near black<br>Medium | 3–4–5–6–days Concentrated at the periphery of the cyst in semicircles or double lines often crossing at right angles in two rows of dots<br>7–8–9–10 days Comparable to earlier stages but more clearly defined<br>11–12–13–14 days Much of the pigment obscured and in some none visible | 50–60 | 15 | 9–37 |
| *P. falciparum* | Blackish<br>Very coarse | 3–4–5–6–7 days Tendency to concentrate at the periphery of the cyst often in double rows but may also be in two straight chains<br>8–9 days Much of the pigment obscured but some grains usually visible<br>10 days Seldom seen | 10–20 | 10 | 8–60 |
| *P. malariae* | Very dark brown to near black<br>Medium | 3–4 5 days Distributed throughout the cyst but some clumping occurs<br>6–9 days Increased clumping<br>10 days and beyond Markedly clumped and situated at the periphery of the cyst In mature oocysts pigment may or may not be visible | About 30 | Minimum 15<br>Maximum 21 | 5–44 |

REFERENCE

## POSITION OF MALARIAL OOCYSTS ON THE STOMACHS OF MOSQUITOES

The distribution of oocysts on the gut wall varies in different species of mosquitoes It is dependent at least to some extent on whether the secretion of the salivary glands of a species of mos qu to agglutinates the blood as do all the *A maculipennis* complex



Fig 12 *A stephensi* Oocysts distributed over the whole of the gut

or whether as with *A stephensi A gambiae* and many other species the salivary gland secretion acts as an anticoagulant (Shute[1])

Usually in *A maculipennis* most of the oocysts are confined to the posterior two thirds of the gut (Fig 13) If however im mediately after taking a blood meal the insect is placed head downwards and kept there for 48 hours (until the ookinetes have penetrated the gut wall) the picture is reversed most of the oocysts being confined to the anterior half of the stomach (Shute[2]) (Fig 14)

In those species of mosquitoes whose salivary glands do not



Fig 13 *A maculipennis* Oocysts confined to the posterior two thirds of the gut



Fig 14 Most of the oocysts confined to the anterior portion of the gut as the result of keeping the insect head downwards for 48 hours after the infective feed

agglutinate their blood meals the oocysts are distributed unevenly throughout the stomach In heavy infections some cysts are often seen at the posterior end of the anterior portion of the organ (Fig 12)

However in all species of mosquitoes oocysts are more numerous on the lower half of the gut This suggests that gravity is the deciding factor (Huff[3]) It is not we believe due to any differences in the varying degree of thickness of the gut wall or to the presence of a peritrophic membrane as has sometimes been suggested (Buxton[4])

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## ROSS S BLACK SPORES

In the past 35 years we have dissected many thousands of anopheles mosquitoes The great majority were found to have oocysts on the stomach wall or sporozoites in the salivary glands but in about 10% no evidence of infection was detected During the whole period these negative mosquitoes must have amounted to several thousands yet we have never found Ross s black spores in negative mosquitoes dissected up to and including the seventh day after the infective feed The spores have been found on numerous occasions in both stomachs and glands of old mosquitoes in which only very scanty sporozoites were detected

Our observations lead us to believe that Ross s black spores are the result of malaria infection and that if on dissecting a mosquito neither oocysts nor sporozoites are found but the spores of Ross are present either on the stomach in the thoracic muscles or in the salivary glands it is evidence of a previous infection When these spores are found on the stomach they may be round and in a solid clump or a bunch of small globular like bodies often banana shaped and light chocolate to nearly black in colour But when they are present in the thoracic muscles or the salivary glands they are always banana shaped and of a deep chocolate colour In our experience too the percentage of a batch infected with these spores is higher at particular seasons of the year In *A maculipennis* the highest percentage occurs in September and

October (James[1]) at least under laboratory conditions We have found them in mosquitoes infected with all four species of human malaria as well as in culicines infected with avian malaria On many occasions only a small percentage of a batch of *A maculipennis* has become infected when fed on one of our patients suffering from *P vivax* and we think it is significant that where Ross s black spores have been found in such circumstances it was only in those showing either oocysts or sporozoites

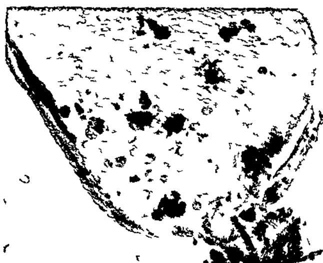


Fig 15 Ross s Black Spores

As regards their origin it would seem that they are chitinised parasites although some workers believe they are chitinised tracheæ (Bruce Mayne ) If however they are chitinised parasites i e oocysts and sporozoites then it is possible that the organisms described by Mayne may be other than the true spores of Ross

When mosquitoes are infected with these spores they can often be seen at the time of dissection with the dissecting microscope Permanent preparations of infected tissue can be prepared by fixing with Bles fluid between the slide and the coverslip dehydrating clearing and mounting in Canada balsam

It is best not to stain the specimen

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## TECHNIQUE FOR COUNTING SPOROZOITES AND INDUCING MALARIA BY INJECTING THEM INTRAVENOUSLY

The transmission of malaria by injecting sporozoites intra venously was first practised by us in 1926 (James Nicol and Shute[1]) During the past 30 years hundreds of patients have been infected by this technique and also from sporozoites from rupturing oocysts which have not come into contact with the salivary glands (Shute )

In 1926 on arriving at the ward of a certain hospital with a jar containing mosquitoes we were called to the telephone before the mosquitoes had fed on the patients On returning to the ward about half an hour later it was found that an enterprising nurse had put the jar of mosquitoes on the radiator to keep them warm All were either dead or moribund and the problem arose how to infect the patients

It was decided to dissect the salivary glands of some of the moribund mosquitoes in Locke s fluid and inject them intra venously This was carried out and all the patients developed malaria within the usual incubation period Because it was found to be completely successful this method of inducing infection has been used extensively and sometimes exclusively by other research workers on simian and avian malaria

It is interesting to record that none of our patients has failed to develop malaria with any of the human Plasmodia following injections of sporozoites from ripe oocysts or from the salivary glands and none has had a protracted incubation period

The procedure is quite safe The slides needles coverslips and Locke s fluid are all sterilised but the mosquito is not because of the risk of killing the sporozoites However on many occasions years ago when we were trying to culture sporozoites by dissecting the infected salivary glands and mixing them with blood the culture often remained free of bacterial contamination for several days even though no special precautions were taken to sterilise the mosquitoes before dissections It seemed as though the secretion of the salivary glands contained some antibiotic properties Duncan[3] has demonstrated that a bactericidal principle occurs in both the gut contents and the fæces of many

insects including mosquitoes It seems likely that this applies also to the secretions of the salivary glands of anopheles

Inoculation of sporozoite suspensions can be useful in several ways In malaria therapy the injection of the salivary glands of one infected mosquito has always proved to be sufficient to induce infection For mass infections the number of sporozoites injected from the salivary glands of 100 infected mosquitoes is at least the equivalent of the bites of a thousand We have proved this on several occasions in the following way

A mosquito was fed on a patient and allowed to gorge itself with blood Within half an hour after feeding its salivary glands were dissected crushed beneath a coverslip and a suspension prepared The sporozoites were then stained and counted If the mosquito was heavily infected (hundreds of oocysts) the number of sporozoites counted often exceeded 200 000 The volunteer who was infected at Horton with *P vivax* to demonstrate pre erythrocytic parasites (Shortt *et al* [4]) was given an injection containing the salivary glands of 200 mosquitoes and a subsequent count of sporozoites proved that at least 15 000 000 were injected All the mosquitoes had fed on the volunteer a few hours earlier thus proving that each mosquito after taking a blood meal still retained about 150 000 sporozoites in its salivary glands This enormous dose of 15 000 000 sporozoites must have contributed to the numerical prevalence of pre erythrocytic forms which were subsequently found in sections of the liver

Each mosquito to be dissected is collected in a rimless 127 mm × 12 7 mm (5 in × ½ in) test tube The head is cleanly severed and the salivary glands are dissected by the technique described in Section 2 As soon as the glands are dissected a drop of Locke s fluid is brought into contact with them The specimen is then brought into focus with the 16 mm (⅔ in ) lens and a square coverslip is applied If the coverslip is dropped on the glands fairly heavily and providing there is not too much Locke s fluid the glands will rupture and release the sporozoites into the fluid Examination with the 4 mm (⅙ in ) lens by reduced light will quickly reveal the presence or absence of sporozoites If the sporozoites are to be injected the coverslip is carefully lifted from the slide without dragging it and a further drop of Locke s fluid is quickly added The fluid is then drawn up in a 1 ml or 2 ml all glass syringe and injected into the animal with as little delay as possible If it is considered advisable to suspend the sporozoites

in serum this should be from the animal to be infected or at least from one of the same species It is however better to dissect the glands in Locke s fluid than in serum because the sporozoites are then more easily sucked up into the syringe If a heavy infection is required i e the glands of hundreds of mosquitoes the material can be pipetted off into narrow tubes until the dissections are completed This calls for a combined operation with 3 or 4 workers dissecting as rapidly as possible with a roving technician equipped with a syringe collecting the suspensions as they become available Because very little is known about the longevity of sporozoites in either serum or Locke s fluid it is best to inject the sporozoites within an hour of dissection We do not know the exact chemical composition of the salivary glands of mosquitoes and although we have had one or two patients who have developed fever and parasitæmia induced by injecting sporozoites which have been in Locke s fluid for 24 hours before being injected we still do not know the percentage of sporozoites which survived for this length of time All we do know is that in this particular case a sufficient number survived to induce infection

Our technique for counting sporozoites is as follows

A 19 mm (¾ in ) square coverslip is fixed to a clean slide with Canada balsam The salivary glands of a mosquito are dissected in Locke s fluid care being taken to obtain all six lobes These are then macerated beneath a coverslip which has been applied so that one corner protrudes over the slide The coverslip is then carefully lifted by holding its corner and raising it so that the fluid and the sporozoites which it contains is concentrated in a small area somewhere near the centre of the slide The coverslip is then washed 3 or 4 times with a few drops of Locke s fluid and this is added to the fluid on the slide The fluid is then taken up into a 1 ml hypodermic syringe and further drops of Locke s fluid are added to ensure that most of the sporozoites are removed This is continued until the quantity in the syringe is exactly 1 ml The contents of the syringe are then discharged into a small flat bottomed watch glass and mixed thoroughly by drawing the material in and out of the syringe a few times Prick the pad of the middle finger of the left hand and with the point of the needle lift off a speck of blood and add to the fluid this gives a suitable background on which to focus when counting sporozoites but only a few hundred cells are required for the purpose

Draw up 0 05 ml of the fluid and blow it on to the square fixed coverslip without spilling any over the slide With a needle distribute the fluid evenly over the coverslip and cover with a petri dish until it is quite dry Fix with methyl alcohol for a few minutes then dry and stain for half an hour with weak Giemsa (one part of stain to 20 parts of distilled water)

**To count the sporozoites**

An Ehrlich eyepiece or its equivalent and a 2 mm ($\frac{1}{12}$ in) oil immersion lens are essential Focus on to the specimen (the scanty red cells present will be helpful here) and move along until the extreme right or left edge of the coverslip is reached Count the number of sporozoites in a horizontal strip and repeat twice at different parts of the coverslip and then strike an average we prefer counting one strip across the top of the coverslip one across the bottom and one across the centre The number of microscopic fields from top to bottom of the coverslip is pre determined by smearing a drop of blood over a coverslip of the size to be used and staining it in the ordinary way the corpuscles enabling one to estimate the exact number of fields It is best to make all counts with the draw tube of the microscope closed

When the average number of sporozoites per strip is known the number of sporozoites in the fluid is calculated If for example there are 100 microscope fields from top to bottom of the coverslip and if the average number of sporozoites per horizontal strip is 100 then the number of sporozoites on the whole of the coverslip is $100 \times 100 = 10\,000$ If the quantity of fluid on the coverslip represents $\frac{1}{20}$th of the whole then the total number of sporozoites equals 200 000 If then the whole of the fluid remaining in the syringe is injected into the patient the total number of sporozoites injected will be 200 000 minus the 10 000 in the 0 05 ml used on the coverslip

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## CULTURE OF THE MALARIA PARASITE

As an aid to the diagnosis of malaria no useful purpose is served by culture methods for there is as yet no practical technique whereby sub cultures increase appreciably the number of parasites following segmentation Furthermore as in three of the four species of human Plasmodium the complete erythrocytic cycle occurs in the peripheral circulation specimens from cultures of these three species would produce nothing which differs from a peripheral blood film However cultures of *P falciparum* trophozoites are of great value for teaching purposes because it is seldom that growing forms beyond the ring stage are seen in the peripheral circulation when they do occur the patient is often gravely ill and drug therapy is a matter of urgency From our experience with *P falciparum* in malaria therapy in primary cases up to about the seventh day of erythrocytic parasitæmia although fever is nearly always quotidian or frequently intermittent the patient is not unduly distressed and rigors seldom if ever occur However beyond the seventh day of fever parasites often occur in the peripheral circulation in enormous numbers often a thousand parasites per twenty five micro fields i e ten per cent of the red cells infected In these cases parasites beyond the ring stage including schizonts are quite common and these parasites are of course heavily pigmented There is a striking difference in the pattern of the pigment of *P falciparum* compared with the same stage of growth in the other three species Instead of a number of separate grains as in *P vivax P ovale* and *P malariæ* in *P falciparum* the pigment is in one two or at most three clumps

Our reason for stressing the above description is that students of malariology should learn to recognise developing *P falciparum* trophozoites in peripheral blood smears because when they occur in numbers in patients undergoing a primary attack the prognosis may be grave

Many workers on malaria who have lived in the tropics for years have told us that they have recognised and appreciated the significance of developing tropozoites of *P falciparum* for the first time when paying a visit to our laboratory

For teaching purposes smears of *P falciparum* parasites can be prepared from cultures which will demonstrate the characteristic pigment as it occurs in trophozoites and schizonts in the internal

*organs* the placenta and occasionally in the peripheral circulation

An alternative to culture smears for obtaining trophozoites and schizonts of *P falciparum* is to make *smears of parasitized* placental blood In pregnant women living in holo or hyperendemic areas the placenta is frequently very heavily infected with *all stages of the asexual cycle* even when parasites are scanty in the peripheral blood Frequently also a large percentage of the leucocytes including polymorphonuclears contains masses of pigment the result of phagocytosis

**Technique for culturing to maturity a single generation of *P falciparum* parasites**

Select a patient in whose blood there are numerous ring stages of the parasite e g at least five parasites per field of a thin film

1 Take 20 ml of venous blood and either defibrinate it or add 160 units of heparin

2 Add 0 2 ml of a 50% solution of sterile dextrose and mix thoroughly with the blood

3 Pipette off into suitable culture tubes to form a column of about 4 c mm and incubate at 37–40°C The parasites live and grow at the top of the column of sedimented red cells and most of the parasites beneath this layer die within a few hours

4 At *intervals up to 36–48 hours of incubation* withdraw a small quantity of blood with a pipette from the top layer of the sedimented cells for examination The culture tubes must never be tilted

5 When *drawing off some of the top layer of blood* for preparing thin films include a little of the serum

Because the asexual cycle of *P falciparum* is completed in about 48 hours it may be thought that parasites *should be cultured for* this length of time in order to obtain fully segmenting parasites This is not so and 24–36 hours is about optimum The reason is that the parasites may be several hours old at the time the blood is taken and before the culture is prepared For class purposes films should be made at frequent intervals so that all stages of growth of the parasite are available for study

## PREPARING SECTIONS AND SMEARS OF POST MORTEM AND BIOPSY MATERIAL

Malaria parasites are very difficult to stain satisfactorily in tissue sections and direct smears give better results Even in smears of

post mortem material the parasites do not stain well unless they are prepared within about three hours after death

It is seldom if ever that a patient dies from an uncomplicated attack of malaria caused by any species other than *P falciparum* When death is directly due to *falciparum* malaria parasite density is generally high and parasites are distributed throughout the vascular organs The brain and spleen are nearly always heavily infected and to a lesser extent the heart kidney lungs and bone marrow As pointed out by Field and Shute[1] parasites are recognised from tissue smears prepared shortly after death but in preparations made several hours after death they are usually unrecognisable and heavy growths of saprophytic bacteria obscure the microscopic picture In smears of this kind the parasites look more like large cocci due presumably to contractions and degeneration Therefore post mortem tissue should be prepared as soon after death as possible The brain and spleen are the two organs most likely to reveal large numbers of parasites malarial pigment and phagocytosis

Where a post mortem is not permissible for religious or traditional reasons some spleen material may be obtained with a syringe and a large exploring needle Brain tissue may be obtained by passing a needle attached to a syringe through the orbital plate of the frontal bone and applying suction The best method we have seen was that demonstrated by Dionisi in Rome many years ago Instead of a syringe he used a cobbler s needle The point of the instrument was skilfully inserted through the conjunctiva under the eyelid and then forced upwards through the orbital plate into the brain The instrument was then revolved two or three times and this filled the eye of the needle with grey matter If this operation is performed carefully there is no disfigurement

In autopsies performed within 24 hours or longer after death most of the parasites have degenerated almost beyond recognition However the pigment remains and this offers the best guide for diagnosis

Some reasons why smears are preferable to sections are

(1) A really thin smear is thinner than a thin section

(2) The parasite stains better

(3) In brain smears the capillaries are well preserved and can be traced for a longer distance than is usually possible in sections

**Technique of preparing and staining smears of brain tissues**

A small piece of brain no larger than the head of a pin is placed in the centre of a dry clean slide A second slide is applied in such a manner as to squash the piece of tissue and drag it over the surface of the first slide exerting continuous pressure In this way two very thin smears are made After the smears are quite dry they are fixed for 5–10 minutes in methyl alcohol (longer than for an ordinary blood film) and are then stained with Giemsa diluted with distilled water 7 ml –100 ml with a pH of 7–7 2 for 30 minutes Flush with distilled water and dry

After the films are dry soak them for 5 minutes in normal saline This removes much of the methylene blue from the tissue cells without decolorising the cytoplasm of the parasites and gives a much clearer picture

After treating the films with saline wash again with distilled water to remove all particles of salt

Dry and examine

**To prepare and stain smears of splenic material**

It is difficult to make very thin films of pulpy splenic tissue and we find it a good plan to break down a small piece in a few drops of saline From this material satisfactory thin films can be prepared

After the films have dried they are fixed for several minutes with methyl alcohol and stained with Giemsa It is our experience that neither Leishman the hæmatoxylins nor the new rapid methods now used for staining thick blood films are as satisfactory as Giemsa for staining parasites in tissue smears Of course the pigment is prominent whatever stain is used but so it is when the film is examined without staining

The technique described above for making and staining smears of brain and spleen is also applicable to other organs heart liver kidney and bone marrow

If it is desired to remove the pigment before staining this can be done after thorough fixation by soaking the film for at least one hour in 1 / HCl in acetone In addition to dissolving the malarial pigment this also dissolves extraneous hæmoglobin without interfering with the cytologic picture

The treated films must be washed in running water for at least 15 minutes to remove every trace of acid before staining

**Fixation and preparation of malaria tissue for staining and sectioning**

The fixative generally used is 10% formal saline This is quite satisfactory for subsequent staining with any of the hæmatoxylins Unfortunately however sections of formalin fixed material cannot be stained satisfactorily with Giemsa If therefore sections are to be stained with Giemsa a fixative not containing formalin must be used Sections of tissue fixed in Carnoy stain beautifully with Giemsa as described by Shortt and Cooper[4] for demonstrating exo-erythrocytic malaria parasites in the liver Their technique is as follows

MATERIALS REQUIRED

Giemsa stain
Acetone and xylol solutions
10%-15% solution of colophonium resin in acetone

Prepare stain as follows

10 ml Giemsa stain
10 ml methyl alcohol
10 ml acetone
70 ml distilled water pH 7 2

After sections are quite dry they should be placed between two pieces of filter paper and rolled flat with a squeegee

(1) Dissolve wax with xylol
(2) Downgrade through alcohols to water
(3) Stain sections for at least 1 hour
(4) Wash in tap water for several minutes
(5) Immerse slide in jar containing colophonium resin acetone solution (The time required for optimum differentiation varies from a few seconds to a minute or even longer depending on the depth of staining)

When the sections are taken out of the staining jar they are a bluish mauve colour But after a few seconds in the colophonium resin acetone solution much of the methylene blue is extracted and they are then a violet pink With practice the exact time to check the decolorising process can be judged with the naked eye but to beginners new to this technique the specimen should be examined under the low power of the microscope However before doing this the specimen should be bathed in 70

acetone xylol solution otherwise a resinous film will form on the section

When the desired degree of differentiation has been obtained the sections are passed through three grades of acetone xylol solutions a few minutes in each

(1) 70% acetone—30% xylol
(2) 50% acetone—50% xylol
(3) 25% acetone—75% xylol

This is followed by two changes in xylol and the sections are then mounted in Euparal vert

*Specimens stained by this technique and mounted in Euparal (not Canada balsam) do not fade for several years*

*The disadvantage of Carnoy as a fixative is that it causes lysis of the red cells As far as we are aware there is no fixative for tissue which fulfils all the requirements for subsequent satisfactory staining by both Giemsa and the hæmatoxylins*

Of all the hæmatoxylins we have tried we prefer Ehrlich s (undiluted) prepared in the laboratory and thoroughly matured Sections are stained for one hour or longer and then bathed for a minute or two in a 1% solution of HCl in 70% alcohol Providing the laboratory tap water is slightly alkaline washing the sections for half an hour is sufficient to blue them An alternative is to soak them in distilled water rendered alkaline by adding a few drops of a saturated solution of lithium carbonate After upgrading in alcohol the sections are counterstained in a 0 01 solution of erythrosine in absolute alcohol for half an hour *This is preferable to counterstaining with a stronger eosin solution for a shorter period They are then passed through 30% phenol xylol cleared in xylol (two changes to remove every trace of phenol otherwise fading quickly occurs) and finally mounted in neutral balsam*

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## SECTION 3

## ESTABLISHING AND MAINTAINING COLONIES OF ANOPHELES IN THE LABORATORY

### GENERAL PRINCIPLES

WHILE it is comparatively easy to raise a single generation of mosquitoes from eggs laid by females collected in the field it is often extremely difficult and with many species quite impossible to maintain succes ive generations continuously in the laboratory One of the earliest records of success we have been able to trace is by Major Harold R A M C [1] who succeeded in raising several generations of *Anopheles maculipennis* at the School of Hygiene at Aldershot The gravid adults were collected from Sandwich Kent At the time he was carrying out his researches it was not known that there are several varieties within the species *A maculipennis* but from personal observations we now know that *atroparrus* is the predominant variety present in Sandwich and it is obvious that Harold s original work was with this form It is interesting to record that at one time when he lost his strain he tried to replace it with specimens of *A maculipennis* collected in the Aldershot area He failed but met with success when he again started with the Sandwich strain The predominant variety in the Aldershot district is *messeæ* and the difference between Harold s failure and success is accounted for by the difference between the two varieties in their ability to copulate in confined spaces in the laboratory *atroparvus* will mate in cages one foot cubed while *messeæ* will not do so in relatively large rooms

Harold s success in breeding several generations of *A maculipennis* in the laboratory was subsequently repeated by many other workers with *A maculipennis atroparvus*

In the tropics Moores appears to have been the first to breed successive generations of *Anopheles gambiæ* in Africa and at the present time many species of Anopheles are being maintained as laboratory colonies in various parts of the world These workers have contributed to our knowledge of mosquito bionomics and it is difficult to see how the present intensive researches on insecticides could be carried out if it were still not possible to breed and

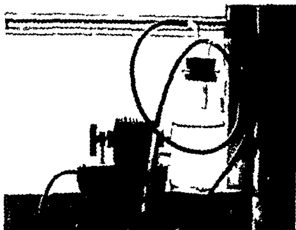


Fig 16 Suction pump used for collecting pupæ



Fig 17 Small bottles containing 10 / glucose with protruding plug of cotton wool as used in store cages to provide food for both male and females

Trays for sand 914 4 mm × 1292 2 mm × 101 6 mm
(3 ft × 4 ft × 4 in )
Long sleeved glove
Dimethyl phthalate

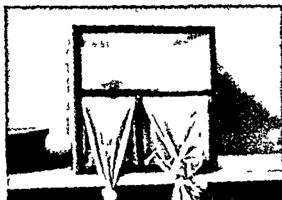


Fig 18 Stock cage containing mosquitoes showing an anæsthetised guinea pig

Cages—cubical—457 2 mm (18 in ) The sides back and front are covered with mosquito netting stretched on a wooden frame The top and bottom are made of three ply wood The bottom half of the front of the cage is divided into two each with a netted sleeve to enable the anæsthetised rabbit to be inserted and also for the introduction of mosquitoes and pupæ jars Parallel with and about 25 4 mm (1 in ) away from the outside of the cage a plastic covered wire is fixed on which damp clothes can be hung to increase humidity A wooden shelf is fixed inside the cage for the egg laying dish and on this shelf two clips are screwed about 114 3 mm (4½ in ) apart to hold the bottles containing the glucose and cotton wool

The colony of *A stephensi* was established in 1947 from eggs sent by Lt Col Jaswant Singh Director Malaria Institute of India

The insectarium used for the breeding of this species is an ex army Nissen hut divided into two parts each with two double windows The room with a south-east aspect is used for the bre d ing of the larvæ because it gives the best possible conditions of natural lighting The adjoining room with a north west aspect is used for housing the adult mosquitoes Both rooms are lined with plaster boards and have concrete floors Heating is supplied by non radiant heat the heat being enclosed in tubular heaters which are in two pairs in each room producing 360 watts per tube The heaters are connected to two thermostats per room and are kept at 25°–27°C The tubular heaters are protected by guards made of perforated metal The light in the room is suppli d by standard daylight tube lighting and each room contains a portable lamp readily adjustable to any position These lamps are of low voltage to ensure perfect safety from shock risks All electrical apparatus is properly earthed in view of the damp conditions Both the room for the breeding of the larvæ and the room for the housing of the adults contain a sink hot and cold water taps and a draining board for cleaning the breeding bowls etc The bowls containing the eggs and larvæ of the colony are accommodated on three tables about 2 m (7 ft) long two under the windows and one opposite the window In the adjoining room the cages containing the adult insects are placed on similar tables The humidity is maintained by a constant drip of hot water from a tap in each room and also there are several trays half filled with saturated sand ballast It is important that the surface of the ceilings and walls be not freshly painted whitewash or an untreated surface is considered satisfactory and is to be preferred If paint has to be used the adult mosquitoes should not be housed in the insectary for at least a month after the painting is completed

**The larvæ**

Success or failure in the rearing of a mosquito colony depends a great d^al on the technique used for raising the larvæ

When the *A stephensi* colony was first started the method used for breeding the larvæ was that described by Shute[1] for the rearing of *A atroparvus* Although this was fairly satisfactory the method used by the East Africa Malaria Unit Amani for the breeding of *A gambiæ* is far superior (Shute G T ) This tech nique is ideal for the breeding of *A stephensi* larvæ and hundreds

of thousands of insects can be obtained with the minimum of effort

TECHNIQUE

White polythene bowls 304 8 mm diameter × 127 mm deep (12 in × 5 in ) are used as rearing pans Fresh distilled water or filtered rain water is added to the bowls to a depth of 38 1 mm (1½ in ) and evaporation is made good by replenishing the water each day The dishes containing the *stephensi* eggs are taken from the cages which house the adults and are allowed to hatch Forty eight hours later 100–150 larvæ are put into bowls containing the distilled water where they are kept until they pupate The pupæ are then transferred to a glass jar which is placed in the cage in which the adults will emerge Larvæ should not be overcrowded in the rearing bowls overcrowding is just as deleterious to the second and third instar as it is to the fourth instar larvæ and an increase in the quantity of food provided cannot compensate for such overcrowding

The larvæ are fed on dried yeast tablets finely ground with a pestle and mortar in a proportion of 1 gramme of yeast per 100 ml of water On hatching 2 ml of the yeast water mixture is pipetted into the egg laying dish On the day when the larvæ are separated 5 ml of the mixture is added to each dish and this procedure is repeated daily until the pupæ develop feeding should then cease The yeast water mixture should be prepared immediately before use as it rapidly deteriorates when left standing The yeast tablets should be kept either in a desiccator or in a dry glass jar with an airtight lid

Every batch of 100 150 larvæ has its appropriate bowl and position on the table according to age and as soon as pupation is complete the bowl is cleaned ready for new larvæ and the next bowl containing larvæ about to pupate is moved into position

The advantage of this method is that all the larvæ develop at about the same rate of growth and pupation is reached within about 48 hours of each other

The pupæ are collected by means of a vacuum pump which is connected to an intake bottle This mechanical collector enables large numbers of pupæ to be collected in a short time The pupæ are transferred from the intake bottle into glass jars and placed in the breeding cages

**The adults**

The majority of *A stephensi* adults emerge in 30-40 hours after pupation when the temperature is 25 -27 C *A stephensi* will mate in a cage of any size but the one described above is the most suitable

The adults for stock purposes are kept in a room facing north west where a temperature of 18 C is maintained and at this temperature the adults are given a blood meal three times per week Either a human arm or a rabbit is used for this purpose The rabbit is anæsthetised by Nembutal injected intravenously intramuscularly or interperitoneally The makers recommend 1 gramme (1 ml ) for each 5 lb weight but it must be remembered that animals have a varying tolerance for the barbiturates some requiring a higher dose than others Nembutal should be given slowly when administered intravenously For intramuscular or interperitoneal injections the same dose is usually employed anæsthesia is complete in the majority of cases in from 10-15 minutes and will last for a period of 1-2 hours For intramuscular injections we inject the Nembutal between the scapulæ

Anæsthetised rabbits are used in preference to guinea pigs for feeding *A stephensi* because a high death rate in the insects occurs in a few hours after they feed on guinea pig blood the death rate is presumably due to the inability of this species of Anopheles to digest the blood meal

We do not shave the hair off any part of the animal though no doubt this would be an advantage if only a few were available

When there are several hundreds of mosquitoes to be fed the anæsthetised animals are placed in the cage on their backs and the mosquitoes feed from the naked parts legs parts of the head the ears and around the anus Neither anophelines nor culicines are able to feed through the very hairy or furry parts of an animal although repeated efforts are made to do so

Nembutal fulfils a very useful role in the insectary With care even very small animals such as 3 or 4 week old chicks can be anæsthetised and immobilised for from 1-4 hours sufficient time to allow hundreds of mosquitoes to feed

When adults are collected by hand from a cage for the purpose of infecting them with malaria or when collecting infective mosquitoes many will bite if the hand remains in the cage for only a few seconds We find it satisfactory to smear the hand and arm with dimethyl phthalate or alternatively to wear a long sleeved glove

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## THE LABORATORY REARINC OF *ANOPHELES MACULIPENNIS* Var *ATROPARIUS*

EQUIPMENT REQUIRED

The apparatus is almost the same as is used for the breeding of the *A stephensi* colony except in the requirements for the breeding of larvæ

- Unglazed earthenware pans 457 2 mm (18 in ) diameter × 114 3 mm (4½ in ) deep
- Unglazed earthenware pans 127 0 mm (5 in ) diameter × 38 1 mm (1½ in ) deep for egg laying
- Rain water
- Jug and funnel for filtering the water Filter made of fine mesh gauze
- Sods of grass
- Farex (Glaxo Ltd London)
- Bemax (Vitamins Ltd London)
- Dog biscuit (Entwhistles Ltd Liverpool)
- Glass jar with screw top for keeping Farex
- Glass jar with screw top and wire cloth centre for Bemax and dog biscuit mixture
- Wire cloth—mild steel 60 × 60 mesh × 37 s w g (Greenings Warrington)
- Close mesh sieve or Vortex hand grinding mill (Bryan Corcoran London)
- Small Hy flo pump for aerating water (Medcalf Bros Florence Works Islington N 1)
- Rabbits or guinea pigs

The colony was started in 1934 from the eggs of a single female caught at Grain in the county of Kent For 9 years prior to this colony being established the adults of *A maculipe nis* var *atroparius* used in the laboratory were caught under natural conditions from an area bordering on the Thames Estuary

The insectarium used for the breeding of this species is s equipped to that alrea ly described for the breeding of A The rooms where the larvæ are bred and the adults

situated on the ground floor of the Malaria Therapy Unit They are of the same brick and concrete construction as the rest of the building

### The larvæ

The method used for breeding *atroparvus* larvæ is based on the work by Shute[1] Three to four hundred larvæ are put into an earthenware pan containing filtered rain water with a small sod

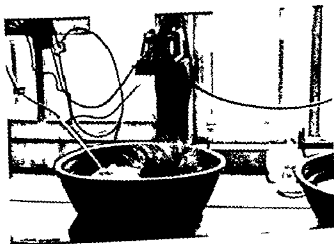


Fig 19 Breeding pan showing the water being aerated by a pump

of grass placed in the middle The mud part of the grass sod is beneficial to the growth of the larvæ and it is quite unnecessary to renew the grass sod or change the water except when fresh bowls are prepared for newly hatched larvæ These pans should be thoroughly scoured with boiling water before preparing a new breeding ground

If the breeding pans have concave bottoms (usual in earthenware vessels) they will not lie flat on the laboratory bench When grass sods are added to the basins sooner or later ants will be introduced Inevitably nests will be made on the bench under the hollows of the pans and the insectarium will soon become infested The pans should rest on bricks or battens and so be raised above the level of the bench by a few inches Ants can be

kept under control and prevented from entering under doors and through crevices by sprinkling a mixture of equal parts of sifted icing sugar and borax around the rooms Ants are partial to sugar and the borax acts as a poison

Rain water is stored in a large tank covered with a metal lid to prevent the breeding of all aquatic insects

The larvæ are fed at intervals throughout the day with either Farex or a mixture of 10 % Bemax and 90 % dog biscuit Farex is an infant food (carbohydrate 73 0 protein 14 0 fat 3 0 mineral salts 4 0 % moisture 6 0 ) it spreads evenly over the surface of the water and floats for a considerable time Bemax is a rich natural vitamin protein mineral supplement (protein 27 % carbohydrates 50 % approximately fibre not more than 3 % calorific value 104 per oz ) and the dog biscuit All in One kennel meal Ten per cent of the Bemax is added to 90 dog biscuit the mixture is passed through a close mesh sieve or a Vortex hand grinding mill and is placed in a glass jar with a screw top In the top of the jar is fixed a very fine wire cloth (60 × 60 mesh × 37 s w g ) and by using this sprinkler the mixture spreads evenly over the surface of the water When not in use the wire mesh top of the jar should always be covered by an inverted petri dish so as to prevent the food from getting damp Should this occur the food will not pass through the fine wire cloth and therefore it should be thoroughly dried and the wire cloth scrubbed and dried also The quantity of food used and the frequency of application depends on the number of larvæ in the pans One should aim at supplying the maximum amount of food that the larvæ will consume but no more than this excess of food will foul the water and cause a high death rate among the larvæ It is best to feed with small quantities at a time and at frequent intervals However should a pellicle form it can be broken up by aerating the water with the Hy flo pump

When breeding Anopheles in the laboratory it is very important to ascertain whether the larvæ are exclusively surface feeders (as *A maculipennis atroparvus* appears to be) or whether like *A gambiæ* and *A stephensi* feeding takes place both on the surface and at the bottom of the breeding pans Larvæ which feed only on the surface must be given food which has the consistency of a powder and which floats on the surface

Large particles of food are rejected by the young larvæ and these particles eventually sink to the bottom of the breeding pan

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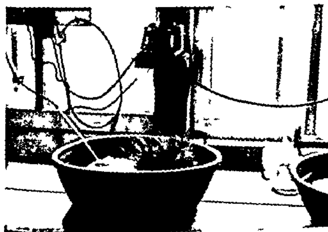


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Large particles of food are rejected by the young larvæ and these particles eventually sink to the bottom of the breeding pan

and cause the water to become sour Therefore it is essential when dealing with larvæ which feed on the surface of the water that the food should be of a dust like consistency

### REFERENCE

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## METHODS OF OBTAINING EGGS FROM INDIVIDUAL *MOSQUITOES*

MATERIALS REQUIRED

Wooden rack 457 mm × 76 mm (18 in × 3 in) Near the top on each side there is a narrow bar which keeps the cylinders in position One of the bars is halved both sections being hinged to the frame This bar is lifted before removing the cylinders from the rack

*Cylinders (glass) 101 mm × 44 4 mm (4 in deep × 1¾ in* diameter)

Petri dishes 38 mm (1½ in diameter)

White card This is cut into pieces 63 5 mm × 63 5 mm (2½ in × 2½ in) and inserted inside the cylinders as a resting place for the mosquitoes without coming into contact with the water in the petri dish

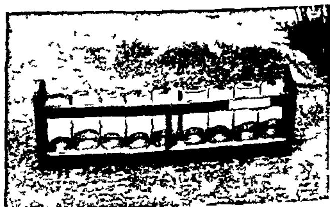


Fig 20 Racks and cylinder jars for obtaining eggs from individual mosquitoes

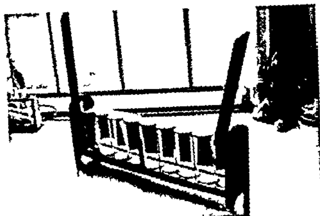


Fig 21 The same w th cross bar raised for remo in cylinders after eg, layin

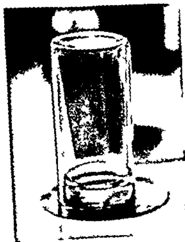


Fig A gra d mosqu to at rest n one of the cyl nders

METHOD 1

If more than one batch of eggs are wanted from an individual mosquito these may be obtained in the following way

A mosquito which is known to be fertilised is given a blood meal and transferred to a glass cylinder which is then put back into the rack over the petri dish of water Eggs are usually laid 2–3 days after the insect has taken a blood meal most often at night After a batch of eggs is laid the cylinder containing the mosquito is carefully lifted from the rack The mosquito is then given a blood meal after which it is again put into the glass cylinder and returned to the rack over a petri dish containing fresh water where it remains until a further batch of eggs is laid Sometimes but not often two blood meals may be necessary to bring the ovaries to maturity

By this method we have succeeded in obtaining 17 batches of eggs from an individual mosquito and some individuals have survived for over 80 days (Shute[1])

*Method 2*

A mosquito with ripe ovaries will nearly always lay its eggs if the following procedure is adopted

*Collect a gravid adult in a 127 mm × 12 7 mm (5 in × ½ in) test tube shake it to the bottom of the tube and rap the bottom end of the tube sharply 7 or 8 times on the palm of the hand to stun the insect (Shute ) When it is immobilised remove it from the test tube carefully remove a wing and transfer it to a petri dish of water Within a few minutes the mosquito will begin to lay its eggs and providing it remains alive laying will continue until all its eggs are laid Care must be taken not to kill the mosquito it should only be rendered sufficiently immobile to enable it to be handled for the purpose of removing a wing to prevent it escaping*

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## LABORATORY INFECTIONS OF ANOPHELES WITH MALARIA

There are four obvious reasons for infecting mosquitoes in the laboratory

(1) For providing infective material for therapy purposes

(2) For providing infective material for heavy infections of sporozoites in the study of the pre and exo-erythrocytic cycle of the parasite

(3) For comparative studies of carrier species of anopheles in a given area

(4) For ascertaining the infectivity to anopheles of the inhabitants in an endemic area

Very little apparatus is required for investigating malaria infections in mosquitoes (Sinton[1] Shute)

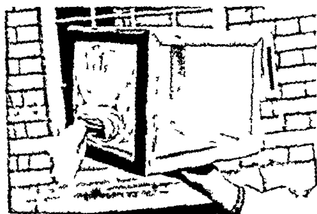


Fig 23 Type of cage used for separating fed from unfed mosquitoes and for storing infected mosquitoes

APPARATUS REQUIRED

(1) A colony of anopheles mosquitoes

(2) Suitable jars for feeding purposes

(3) Test tubes 127 mm × 12 7 mm (5 in × ½ in)

(4) Cages 304 8 mm × 203 2 mm (12 in × 8 in) The sides back and front are covered with white mosquito netting stretched on a wooden frame with a false ceiling about 12 7 mm (½ in) below the true ceiling to prevent the mosquitoes hiding in the corners The front of the cage is provided with a netted sleeve to enable the anæsthetised animal to be inserted and for the introduction of mosquitoes We consider this size cage to be safer for the

housing of the infected insects than the one described for housing the uninfected mosquitoes and makes it much easier to collect them

The technique for reasons (1) and (2) is approximately the same but for (3) and (4) some modifications are required and these will therefore be described separately

For the provision of heavily infected mosquitoes the three essentials are

(1) A suitable animal in whose blood there are numerous mature gametocytes which have previously been tested for ripeness as described in Section 2

(2) Very heavy infections i e several hundreds of oocysts per gut

(3) Over 90% of a batch infected as the result of a single feed

These conditions are only obtained when the blood film shows 200 or more exflagellating male gametocytes per c mm (approximately 5–10 males per 100 leucocytes in an animal showing about 6 000 leucocytes per c mm )

**The feeding jars**

The most suitable jars we have found are made of thick glass cylindrical with a heavy flanged mouth and measuring 88 9 mm (3½ in ) in diameter and height (James[1, 2]) In the bottom of each jar is placed a closely fitting disc of dry filter paper to absorb any moisture which may accumulate during the process of feedin especially if the patient is sweating Over the mouth of each jar is tied with tape a piece of mosquito netting which has an opening in the centre a little larger than the diameter of the test tube used for collecting mosquitoes The insects are captured in a test tube and transferred through the opening into the jar When about 50 mosquitoes have been inserted into the jar the top is covered with another layer of netting which is tied in position with a second piece of tape With some species of mosquitoes such as the comparatively sluggish *A maculipennis* ten or even twenty specimens are easily collected in a single tube But with some other species such as *A stephensi* or *A gambiae* it is difficult to collect more than two or three in a tube at a time As the test tube containing the mosquitoes has to be inserted several times into the hole through the netting covering the top of the jar the second piece of netting without a hole in the centre is used as a plug to prevent escape until all the mosquitoes have been bottled

then the top layer of netting is tied down We prefer white netting both for our cages and for the tops of the jars Also we use an all purpose netting which has 150 holes to 6 4 sq cm (square inch) This is suitable for dealing with relatively large mosquitoes such as *A maculipennis* and also for the small species such as *A stephensi* and *A gambiæ* We prefer test tubes for collecting mosquitoes from the cages rather than the sucking tube used by many workers in the field When mosquitoes are being infected for use after the



Fig 24 Jars used for feeding mosquitoes on humans

completion of the sporogonic cycle (sporozoites in the salivary glands) every care must be taken not to injure them at any stage and especially at the time of infecting them If the sucking tube is used many of the mosquitoes are damaged and many lose one or more legs and also the wings are often damaged

To infect a batch of mosquitoes from a human gametocyte carrier we proceed as follows

The patient lies on his back and is made comfortable so that he will remain quite still The leg is bared from the thigh to the knee and 4 jars as described above are pressed firmly against the skin The person responsible for feeding the mosquitoes should also make himself comfortable so that he can press the jars against the skin and remain quite steady for at least 20 minutes

is best not to provide water for egg laying because they drown very easily However great care must be taken to ensure a constant high humidity (70–80%) otherwise there will inevitably be a high death rate

If more than one species of parasite or more than one strain of parasite is being maintained the ideal is to have a separate room for each This we would suggest is essential where more than one strain of the same species is maintained especially if there are no morphological characters to differentiate between the parasites of strains either in the oocysts in the mosquito or in the parasites in the peripheral blood of the vertebrate host It is a good plan to paint the woodwork of the cages with a distinctive colour for each species of parasite

The technique of infecting large batches of mosquitoes from monkeys birds and other animals is the same as that already described except that instead of collecting the mosquitoes into jars and feeding them by hand the animal is either anæsthetised or otherwise immobilised so that it cannot move about inside the cage

When investigating the comparative susceptibility to infection between several local species of Anopheles all should be fed on the same patient at the same time This may be difficult and call for much patience because it necessitates the availability of large numbers of Anopheles at the time when gametocytes are present in the peripheral blood As the normal death rate among mosquito colonies is usually high not less than 50 mosquitoes of each species should be fed Normally 50–70% will survive for about 7–8 days which means that 20–30 are available for dissection on or about the seventh day (Shute and Maryon[7]) In many experiments of this kind which we have conducted it has been found that the seventh day is about optimum for dissection At this stage of growth with reduced light oocysts are readily detected with the 16 mm (⅔ in) lens and No 6 eyepiece If 20–30 mosquitoes of each species are dissected a fairly accurate comparison of susceptibility to infection of species is arrived at

One of the most striking and perhaps surprising findings in this laboratory has been the inconsistency between gametocyte density and mosquito infectivity For example on many occasions we have found that mosquitoes have become infected when gametocytes have been difficult to find in thin or even in thick films and indeed on some occasions when none has been found

even after a prolonged search This has caused us to consider the problem from the epidemiological angle and to consider how much importance can be attached to gametocyte surveys in the field During surveys an individual blood film is examined for one supposes 2–3 minutes If a worker spends 4 hours at his microscope he will examine 80–100 films From these he may find one or more gametocytes in 5–10 / of the population This would suggest that about 10 in every 100 individuals are infective and from our experience this could be inaccurate It seems to us that gametocyte surveys based on blood film examinations would not give such accurate information as sample feedings of a carrier species If in surveys of this kind about 50 mosquitoes were fed on a percenta_e of the population according to age groups and the surviving insects dissected on about the seventh day a far more accurate picture of the percentage of the population who are at a given time capable of infecting mosquitoes would be arrived at

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## *SECTION* 4

## *PRACTICAL HINTS*

When preparing blood films for teaching purposes no departure from the usual routine is necessary but because the specimens will be required to last for an indefinite period precautions must be taken to delay fading

Adjust the distilled water so that it is neutral or very slightly alkaline after it has been exposed to the atmosphere of the laboratory for 15–20 minutes

If possible films should be stained on the day they are taken but in any case not later than 48 hours afterwards

Unmounted films probably keep slightly better than those which are mounted in a neutral mountant but if used repeatedly unmounted films soon get scratched or damaged

Never use Canada balsam for mounting films stained with Leishman or Giemsa stain

Euparal vert is excellent and specimens mounted in this medium often fade very little after 20 years or more

If films are not mounted the immersion oil should be thoroughly dissolved with neutral xylene before wiping the film To put xylene on the oil and then to wipe the film before the oil is thoroughly dissolved quickly ruins the film

Xylene is sometimes strongly acid especially when it is old or when the bottle is only half full for long periods

Only No 1 coverslips should be used for mounting films which are subsequently to be examined with an ion lens

Films when ild be ark and if possible in a *dry* a des g calcium chloride) and n

Thick films unstained for a week or more should be soaked in normal saline before being stained especially in the tropics The length of time a film should be soaked in saline depends on its age and thickness The aim should be to continue soaking the film in saline until most but not all of the hæmoglobin is removed The film should then be stained while wet Very thick films even though they may have been taken only a few hours previously should be soaked before staining

All stains and especially the Romanowsky modifications Leishman and Giemsa should be prepared in the laboratory and not purchased in solution

Stock solutions of Giemsa and Leishman stain should be kept away from strong light It is a good plan to cover the vessels containing the prepared stains with brown paper held in position with rubber bands

Because heat also plays a part in stain deterioration stock solutions should be kept at temperatures below 16 C

For diluting Giemsa measure by millilitre and not by drops This prevents confusion concerning the number of drops to a millilitre

Thick and thin films will stain perfectly up to at least 3 years if kept in a desiccator which is stored in a domestic refrigerator

Thick and thin films which are to be stored in a desiccator for subsequent staining should be prepared from freshly drawn blood and *on no account should these films be fixed prior to storing* Films prepared from defibrinated or citrated blood do not stain well even after a few days Films from heparinised blood stain satisfactorily several weeks after they are prepared

A more even thick film is obtained if the blood is distributed on the slide to form a square instead of a circle and the staining is more uniform

When thick and thin films are made on the same slide great care must be taken to prevent any alcohol from touching the thick

film If this happens the latter will not dehæmoglobinise The thick film should be made about 12 7 mm (½ in ) from one end of the slide the thin film should be spread in the opposite direction a gap of about 12 7 mm (½ in ) being left between the two

It frequently happens in the tropics that absolute alcohol is tampered with by irresponsible employees It is therefore advisable to test the alcohol before use when preparing fixatives etc The best method is to use a hygrometer where this is not available a good indicator is copper sulphate Industrial spirit 74 O P is 99 per cent alcohol and is much cheaper than ethyl alcohol

The relatively new method of producing mineral free water by passing it through a deioniser has much to recommend it Once the apparatus is available the cost of maintaining mineral free water is cheaper than producing distilled water and what is more important it is the equal of treble distilled water This deionised water is excellent for rearing mosquito larvæ by the method described in this book for rearing *A stephensi* It is also excellent for diluting alcohol and all water solvent stains

If distilled water is boiled in a soft glass flask it becomes strongly alkaline Always test the pH of distilled water which has been boiled before using it for diluting Romanowsky stains

If the distilled water is too alkaline never add acid for adjusting Instead add distilled water which has become acid through exposure to CO

Perfect thin films cannot be made with thick slides or even with thin ones which have chipped edges Always keep a few extra thin glass slides with clean bevelled edges for preparing thin films

Instead of coating a paraffin block with a low melting point wax to ensure a ribbon raise the temperature of the room where the sections are to be cut When sections separate from each other as they leave the knife it is often due to the temperature of the room being too low Although this can be corrected by coating the paraffin block with a low melting point wax thishas the disadvantage of causing the sections to become widely spaced when they are heated for flattening on the slide

Mosquitoes which are to be killed and fixed for sectioning should not be fed on sugar solutions for at least 3 days before killing them When the diverticulum is distended with fluid it presses against the mid gut and causes distortion

When only a thin film is available for examination and parasites are not found easily with the 2 mm ($\frac{1}{12}$ in) oil immersion lens change to a 3 75 mm fluorite lens Although magnification is thereby reduced by nearly one half the smallest *P falciparum* ring can be seen quite clearly The 3 75 lens is also useful when searching for scanty gametocytes With the modern high grade binocular microscopes a 3 75 lens is adequate for all routine blood film examinations of both thick and thin films and is a great time saver Alternatively a 4 mm ($\frac{1}{6}$ in) dry lens with a No 6 or No 10 eyepiece can be used It is necessary however to cover the specimen with a film of oil and apply a No 1 coverslip

Stomachs and sections of mosquitoes showing oocysts stained with hæmatoxylin and mounted in a neutral medium usually do not fade perceptibly over several years If fading has occurred however valuable specimens can be restored by the following technique

(1) To remove the coverslip soak the slide in xylol if the specimen was mounted in Canada balsam or in Euparal essence if it was mounted in Euparal This usually takes several days

(2) Absolute alcohol (two changes) at half hourly intervals

(3) One per cent HCl in 70 alcohol and leave until all the stain is removed (usually 1–2 hours)

(4) Running water for at least one hour to remove all traces of HCl

(5) Stain for about 10 seconds with Mayer s hemalum (Pantin s)

(6) Blue in alkaline water

(7) Upgrade in alcohols to absolute (74 O P Industrial Spirit) a few minutes in each

(8) Carbol xylol in 30 phenol in xylol (30 minutes)

(9) Two or three changes in xylol to remove all traces of phenol

(10) Mount in a neutral medium

If difficulty is experienced in starting a laboratory colony of a *species of mosquito whose habits are unknown* the first essential is to ascertain whether copulation occurs in the laboratory Remove one or two females from the cage after they have been in contact with the males for a few days and dissect out the spermotheca *For this dissection use the white background of the* dissecting microscope because then the dark brown spermotheca will show up clearly Dissect the spermotheca in saline transfer the specimen to the microscope and apply a coverslip under pressure sufficient to rupture the sac Examine with the 4 mm ($\frac{1}{8}$ in ) lens If positive the sperms will be seen swarming from the ruptured sac Even in an unruptured spermotheca the sperms can often be seen and there is usually a rotary anti-clockwise movement of the sperms within the sac

To observe the process of egg hatching lift an egg from the surface of the water with a triangular piece of filter paper selecting one that has been laid about 36 hours Transfer it to a cavity slide containing 10 / formalin and apply a coverslip In a few minutes the larva will emerge and the formalin will kill it It can then be mounted as a fluid specimen or the formalin can be pipetted off and a different mountant used

When injecting animals with Nembutal wipe the needle to prevent the drug leaking This will prevent sores developing at the seat of injection

The points of dissecting needles soon become damaged unless special care *is taken to protect them* even sticking them in a cork can be harmful Keep the needles blades downwards in a test tube which has a cotton wool plug at the bottom and a stopper at the top If ordinary steel needles are used they should be *washed in hot water at the end of the day s* work to remove all traces of salt and then thoroughly dried to prevent rust forming

*Slide dryer slab* When large numbers of slides are to be *stained in bulk they can be set up to dry in* the following way A wooden slab preferably of teak if made to the following measurements will carry half a gross of slides

240 mm × 240 mm × 18 mm ($9_{16}$ in × $9\frac{7}{16}$ in × $\frac{3}{4}$ in )
*The slab is divided into nine lines of grooves* each 24 m apart

The grooves are 2 m wide and 3 mm deep By having the grooves twice the thickness of the slide it is ensured that the slide will rest at a slightly slanting angle If the slides are placed in the grooves with the blood film underneath they will remain dust free while drying If the lines are too close to each other or if the grooves are too wide the slides when at rest will touch each other and so prevent them drying Plastic material prepared to

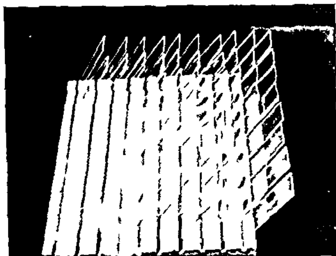


Fig 25 Slide dryer slab to hold half a gross of sl des

the above measurements would probably be as satisfactory as teak and of course would be much lighter

Hardening and brittleness of tissue can be avoided if after the tissue has been in alcohol for not more than 2 hours it is then transferred to methyl benzoate for 24 hours or longer Transfer to benzene two changes 1 hour in each and then into paraffin in the usual way

Many substitutes for cedar oil immersion lenses are being used extensively these days Anisol is satisfactory and has the advantage

that it is not sticky or greasy It does not harden on the slide and a drop of Anisol disappears from the slide in about 20 minutes In the humid tropics immersion oils quickly take up water vapour Also the moist breath of the microscopist can cause water vapour to reach the film and make examination tedious and difficult

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